Title: C-type lectin binding molecules, identification and uses thereof.

The invention relates to the field of immunology. The invention in particular relates to the role of dendritic cells in immune responses and to pathogens that are able to capitalize thereon.

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Dendritic cells (DC) are professional antigen presenting cells (APC) that induce cellular immunity upon pathogen recognition. These cells are therefore important in the defense against many pathogens [1;2;3]. Immature DC are seeded throughout peripheral tissues to act as sentinels against invading

10   pathogens[4]. Upon pathogen capture, DC are activated, process pathogens for antigen presentation on Major Histocompatability Complex (MHC) class II molecules, and migrate to the secondary lymphoid organs where they activate naïve T cells to initiate adaptive immune responses[1;3;4]. Depending on the pathogen that is recognized by the DC, differentiation of naïve T cells into Th1

15   cells is triggered by DC in response to intracellular microbes, whereas Th2-mediated responses are generated by DC to eliminate pathogens residing extracellularly [3]. Thus DC play an important role in both innate and cellular immune responses against tumors antigens as well as pathogens such as viral, bacterial, fungal and parasitic infections[1;5]. Knowledge about cell-surface

20   receptors on DC that are involved in recognition of pathogens is only starting to emerge, and include Toll-like receptors (TLR) [6;7] and C-type lectins [8]. TLR recognize specific pathogen-derived components, such as lipoproteins, lipopolysaccharides and bacterial DNA, and relay this information through intracellular signaling cascades leading to the production of regulatory

25   cytokines and upregulation of MHC and costimulatory molecules that lead to activation/maturation of DC[6]. In contrast, C-type lectins recognize pathogen-derived carbohydrate structures and upon binding internalize pathogens for antigen processing and presentation to T cells[8-10]. In classical calcium-dependent lectins, conserved amino acid residues in the carbohydrate

2

recognition domain (CRD) are involved in calcium binding and sugar specificity [11]. A growing number of C-type lectins are described to be specifically expressed by DC. For most of these lectins detailed knowledge about pathogen-targets as well as cellular ligands if any, including the identity

5 of the carbohydrate structure they recognize, is lacking[8]. The DC-specific C-type lectin DC-SIGN (dendritic-cell specific ICAM-3 grabbing nonintegrin, CD209) is involved in binding of the HIV-1 envelope glycoprotein by DC to enhance infection of T cells [12], while the mannose receptor (MR) is involved in recognition of mycobacteria and Fungi/Protozoa [13]. Some C-type lectins like

10 DC-SIGN can interact with carbohydrate-bearing self glycoproteins (ICAM-2 and ICAM-3) to mediate cellular adhesion processes [14;15].

In one aspect of the present invention it was found that a C-type lectin can comprise specificity for more than one type of glycoconjugate. This

15 knowledge is useful for a variety of purposes. For instance, lectins are instrumental in mediating pathogen binding and the presentation of antigens thereof in dendritic cells. This binding property of C-type lectins is utilized by a number of pathogens to at least in part facilitate infection of an individual. Knowledge of glycoconjugate specificity of such lectins therefore provides an

20 entrance to the development of medicaments capable of interfering with the capacity of carbohydrates present on pathogens to interact with the lectin and thereby at least in part interfere with the infection or the severity thereof in the exposed individual. In one embodiment the invention therefore provides a method for at least in part inhibiting the binding of a ligand to a C-type lectin

25 or a carbohydrate binding part thereof, comprising contacting said C-type lectin with an isolated and/or recombinant glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or a glycoconjugate comprising at least one end standing N-acetylglucosamine or a derivative or multimer thereof. Also provided is the use

30 of a glycoconjugate comprising at least two mannose residues in α1,2 linkage

or a glycoconjugate comprising a fucose residue or a glycoconjugate comprising
at least one end standing N-acetylglucosamine or a derivative or multimer
thereof for at least in part inhibiting the binding of a ligand to a C-type lectin
or a carbohydrate-binding part thereof. This method may be used for instance

5   to study the exact binding properties of the lectin. The method is also of use in
identifying compounds capable of interfering with an ability of said C-type
lectin to bind to pathogens. In a preferred embodiment said method further
comprises a cell comprising said C-type lectin. Preferably said cell is an
antigen presenting cell, more preferably a dendritic cell or macrophage. In the

10   presence of such cell, the effect of such inhibition may be compared to the
function of the antigen presenting cell, for instance on its capacity to present
antigen and/or the production of lymphokines and/or cytokines. In a preferred
embodiment said C-type lectin comprises DC-SIGN, mSIGNR1, L-SIGN and/or
DC-SIGNR. Whereas DC-SIGNR and L-SIGN are predominantly expressed on

15   macrophage/endothelial cells, on lymph node and on liver sinusoidal
endothelial cells and DC-SIGN is expressed predominantly on dendritic cells.
DC-SIGNR and L-SIGN share the remarkable binding and signaling effects
that ManLAM has upon binding to dendritic cells via DC-SIGN. However,
whereas DC-SIGN further comprises a particular specificity for

20   glycoconjugates comprising a fucose residue or a derivative or multimer
thereof, DC-SIGNR and L-SIGN are lacking such particular specificity. In the
present invention there is thus a preference for the C-type lectin DC-SIGN.

With the term "glycoconjugate comprising a fucose residue" is meant a

25   glycoconjugate comprising at least one fucose residue that is α1, 3 or α-1,4-
linked to the glycoconjugate. The linking moiety preferably consists of N-
acetylglucosamine. The linking moiety is preferably coupled to an
oligosaccharide or glycan, which in turn may be part of a larger structure (or
carrier molecule) comprising subsequent glycoconjugates comprising a fucose

30   residues, or other compounds such as mannose in any configuration. A

4

derivative of said glycoconjugate comprising a fucose residue comprises the same C-type lectin binding activity in kind not necessarily in amount. A derivative may be generated through modification of the fucose residue. Such modifications may be generated in various ways. Preferably, said fucose is a

5   terminal fucose, i.e. linked via only one $\alpha 1$, 3 or α-1,4 linkage to another molecule or fucose. Preferred examples of glycoconjugates comprising a fucose residue are Lewis bloodgroup antigens Le^x, Le^y, Le^a, Le^b and/or LDNF. C-type lectin binding parts, derivatives and/or analogous of the lewis bloodgroup antigens are of course also within the scope of the invention. Different

10   modifications have different effects on the lectin binding properties. Sialylation of Le^x (yielding sialyl-Le^x, a L-, E- and P-selectin ligand) completely abrogates the recognition by DC-SIGN, indicating that DC-SIGN has a carbohydrate specificity that is distinct from that of the selectins that mediate leukocyte rolling. Sulfation reduces the binding affinity of DC-SIGN for Le^x, as well as

15   Le^a (Figure 2a). A derivative of a glycoconjugate comprising a fucose residue thus comprises a modification wherein said modification allows binding of said glycoconjugate to said C-type lectin. Glycoconjugates comprising multiple fucose residues in the configuration of a lewis bloodgroup antigen typically have a higher binding affinity than a glycoconjugate comprising a single fucose

20   residue.

With the term "glycoconjugate comprising at least two mannose residues in $\alpha 1,2$ linkage" is meant a glycoconjugate comprising at least two mannose residues wherein said mannose residues are linked to each other via an $\alpha 1,2$

25   linkage and wherein one of the mannose residues is linked to the glycoconjugate. The linking moiety preferably but not necessarily comprises mannose in α-1,5 linkage and arabinan or β-D-arabinose. The linking moiety is preferably coupled to an oligosaccharide or glycan, which in turn may be part of a larger structure (or carrier molecule) comprising subsequent

30   glycoconjugates comprising at least two mannose residues in $\alpha 1,2$ linkage, or

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other compounds such as fucose in any configuration. A derivative of said
glycoconjugate comprising at least two mannose residues in α1,2 linkage
comprises the same C-type lectin binding activity in kind not necessarily in
amount. A derivative may be generated through modification of one or both of

5   the mannose residues. Such modifications may be generated in various ways.
Preferably, said at least two mannose residues in α1,2 linkage are terminal
residues, i.e. linked via only linkage to another molecule or mannose. In a
preferred embodiment said glycoconjugate comprising at least two mannose
residues in α1,2 linkage, comprises two or three mannose residues linked to

10   each other via respectively one and two α1,2 linkages. A particularly preferred
example of a of glycoconjugates comprising at least two mannose residues in
α1,2 linkage is ManLAM. ManLAM comprises a mannose-capped
lipoarabinomannan, such as derivable from the cell wall component of
Mycobacterium. The mannose-capped lipoarabinomannan is preferably linked

15   to an oligosaccharide or glycan, which in turn may be part of a larger structure
(or carrier molecule) comprising subsequent mannose-capped
lipoarabinomannans or other compounds such as fucose in any configuration.
C-type lectin binding parts, derivatives and/or analogous of ManLAM are of
course also within the scope of the invention. Different modifications have

20   different effects on the lectin binding properties. Glycoconjugates comprising
two or more sets of at least two mannose residues in α1,2 linkage typically
have a higher binding affinity than a glycoconjugate comprising only one set of
at least two mannose residues in α1,2 linkage.

25   With the term "glycoconjugate comprising a N-acetylglucosamine residue" is
meant a glycoconjugate comprising at least one end-standing N-
acetylglucosamine residue that is linked to the glycoconjugate. The N-
acetylglucosamine is preferably directly linked to an asparagin of the
glycoconjugate. The glycoconjugate can in turn be part of a larger structure (or

30   carrier molecule) comprising subsequent glycoconjugates comprising N-

6

acetylglucosamine residues, or other compounds such as mannose or fucose in any configuration. A derivative of said glycoconjugate comprising a N-acetylglucosamine residue comprises the same C-type lectin binding activity in kind not necessarily in amount. A derivative may be generated through

5   modification of the N-acetylglucosamine residue. Such modifications may be generated in various ways. Preferably, said N-acetylglucosamine is a terminal N-acetylglucosamine, i.e. linked to the asparagin. Preferred examples of glycoconjugates comprising a N-acetylglucosamine residue. A derivative of a glycoconjugate comprising a N-acetylglucosamine residue thus comprises a

10   modification wherein said modification allows binding of said glycoconjugate to said C-type lectin. Glycoconjugates comprising multiple N-acetylglucosamine residues typically have a higher binding affinity than a glycoconjugate comprising a single N-acetylglucosamine residue.

15   A glycoconjugate can be mono- or polyvalent for the respective mannose, fucose or N-acetylglucosamine residues. A polyvalent glycoconjugate comprises at least two separate antennae wherein at least two said antennae comprise said residues. In a preferred embodiment a polyvalent glycoconjugate comprises at least two antennae having a terminal fucose, i.e. linked via only one α1, 3 or α-

20   1,4 linkage to the glycoconjugate or to another fucose residue. In a preferred embodiment a polyvalent glycoconjugate comprises at least two antennae having at least two mannose residues in α1,2 linkage. In yet another embodiment a polyvalent glycoconjugate comprises at least two antennae having a terminal N-acetylglucosamine, i.e. linked via only one linkage to the

25   glycoconjugate. A multimer is preferably a polyvalent glycoconjugate

A binding molecule can be specific for a monovalent or a polyvalent glycoconjugate. Binding of the binding molecule is typically stronger to the polyvalent glycoconjugate.

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Binding of the C-type lectin and more in particular, subsequent action of the cell carrying the C-type lectin is typically significantly higher in case of binding of a polyvalent glycoconjugate. This is in particular so for glycoconjugates comprising at least one end standing N-acetylglucosamine

5   residue.

Carrier molecules may further comprise a large variety of different molecules including peptide, protein, lipid, polysaccharide and also synthetic molecules not expressly mentioned here. The carrier molecule(s) may of course be part of

10   an even larger structure such as a biological or artificial surface, a virus, a bacterium or a eukaryotic cell.

Binding of the ligand to the C-type lectin can be inhibited by interfering with binding site on the ligand, with binding site of the C-type lectin or both.

15   Interference with the binding site of the ligand can be done using a ligand binding molecule capable of specifically binding to the C-type lectin binding part on the ligand. This is preferably done with a proteinaceous molecule such as a carbohydrate specific antibody preferably specific for a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate

20   comprising a fucose residue or a glycoconjugate comprising at least one end standing N-acetylglucosamine or a derivative or multimer thereof. The experimental part describes non-limiting examples of such antibodies. Non-limiting examples of antibodies comprising a specificity for a glycoconjugate comprising a fucose residue or a derivative or multimer thereof are SMLDN1.1

25   or SMFG4.1, 6H3, Hp151, 4D2, 54.1F6A, NAM61-1A2 or SMLDN1.1. The ligand binding molecule can also be a C-type lectin, preferably a soluble derivative thereof, comprising specificity for a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or a glycoconjugate comprising at least one end standing N-

30   acetylglucosamine or a derivative or multimer thereof. A non-limiting example

8

of a soluble C-type lectin of the invention is a soluble DC-SIGN-Fc chimeric molecule comprising amino acid residues 64-404 fused at the C-terminus to a human IgG1-Fc fragment as described for instance in Geijtenbeek et al (Geijtenbeek et al 2002, J. Biol. Chem. 277:11314-11320). However, soluble C-

5   type lectins comprising one or more of the provided specificities can be generated in various ways (for instance by derivation from DC-SIGNR or L-SIGN). Now that the present invention describes one chimeric molecule others can be generated, for instance as described in Fawcett J et al (Fawcett J et al 1992, Nature 360:481-4).

10   Interference with the binding site on the C-type lectin can of course also be done in various ways. Interference with the binding site on the C-type lectin can be done using a C-type lectin binding molecule capable of specifically binding to the ligand binding part on the C-type lectin. This is preferably done with a proteinaceous molecule such as a C-type lectin specific antibody or with

15   a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or a glycoconjugate comprising at least one end standing N-acetylglucosamine or a derivative or multimer thereof, wherein said glycoconjugate or both may be part of a larger structure. Binding can of course further be inhibited by sterically hindering the binding

20   of the ligand to the C-type lectin. For this it is not absolutely required that the interfering molecule itself binds to the association site of the carbohydrate and the C-type lectin. Binding can also be interfered with successfully steric hindrance by binding of the interfering molecule in the vicinity of the site of association.

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The ligand may be any type of structure capable of binding to said C-type lectin. Usually this will comprise a proteinaceous molecule or a carbohydrate. The ligand may be an antibody. It is preferred, but not strictly necessary, that the ligand comprises mannosilated glycans such as cell wall component of

30   Mycobacterium ManLAM, or a mannose derivative, lipophosphoglycan such as

9

derived from Leishmania, or a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising at least one end standing N-acetylglucosamine or a glycoconjugate comprising a fucose residue such as SEA or CD66 or a derivative or multimer thereof. The ligand can have

5   instead of, or in addition to, a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose or a glycoconjugate comprising at least one end standing N-acetylglucosamine or a derivative or multimer thereof, different glycoconjugates capable of binding to the same on the C-type lectin.

10   The ligand preferably comprises an antigen. The ligand can, as mentioned above, comprise a glycoconjugate comprising a fucose residue, at least two mannose residues or at least one end standing N-acetylglucosamine. In this embodiment the antigen does or does not comprises said glycoconjugate. In a preferred embodiment the antigen comprises said glycoconjugate. The ligand

15   preferably comprises a (tumor) antigen, a pathogen and/or a cell associated receptor. An antigen is here used to include peptides or glycolipids and derivatives thereof capable of being presented in the context of MHC class I or class II, or CD1b. At least some C-type lectins, and particularly those expressed on dendritic cells are involved in the process of antigen uptake and

20   presentation thereof by antigen presenting cells. An early step in this process is the capture of the antigen by such lectins. With a method of the invention it is possible to at least in part interfere with this initial step in the process of antigen presentation. Considering the importance of antigen presentation in the initiation and maintenance of antigen specific immune responses, it is

25   clear that the method of the invention can be favorably used to at least in part diminish the potency of an immune response against said antigen.
        In a particularly preferred embodiment the ligand comprises a pathogen or a C-type lectin binding part thereof. It has been found that many different pathogens comprise carbohydrates capable of binding to C-type lectins of the

30   invention. In particular it has been found that pathogens or parts thereof can

bind to said C-type lectins through a mannose containing glycoconjugate such as a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose or a glycoconjugate comprising at least one end standing N-acetylglucosamine or derivative or multimer thereof. This

5　binding at least in part facilitates infection of an individual with said pathogen. Interference with the binding of the pathogen or part thereof to the C-type lectin can thus at least in part aid an individual in combating an infection by the pathogen and in cases even prevent the establishment of a clinically visible symptoms thereof. The latter is useful in (passive or active)

10　vaccination strategies. The pathogen can be a virus, a fungus, a (myco)bacterium and/or a parasite. The pathogen preferably comprises a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or a glycoconjugate comprising at least one end standing N-acetylglucosamine or a derivative or multimer

15　thereof. Examples of preferred pathogens are human immunodeficiency virus, a *helicobacter*, a *neisseria meningitidis*, a *leishmania*, a *schistosoma*, a *klebsiella*, a probiotic lactobacillus, hepatitis C virus, a herpes simplex virus or an ebola virus. Inhibition of binding of the specifically mentioned pathogens can be achieved using a glycoconjugate comprising at least two mannose

20　residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or a glycoconjugate comprising at least one end standing N-acetylglucosamine or a derivative or multimer thereof. In a particularly preferred embodiment inhibition of binding is achieved using a ligand (pathogen or part thereof) binding molecule capable of specifically binding to the C-type lectin binding

25　part on the ligand. This is preferably done with a proteinaceous molecule such as a carbohydrate specific antibody preferably specific for a mannose carbohydrate such as a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or a glycoconjugate comprising at least one end standing N-acetylglucosamine or a

30　derivative or multimer thereof. The experimental part describes non-limiting

examples of such antibodies. The ligand binding molecule can also be a C-type lectin, preferably a soluble derivative thereof, comprising specificity for a mannose carbohydrate such as a glycoconjugate comprising at least two mannose residues in $\alpha 1,2$ linkage or a glycoconjugate comprising a fucose

5 residue or a glycoconjugate comprising at least one end standing N-acetylglucosamine or a derivative or multimer thereof. A non-limiting example of a soluble C-type lectin of the invention is a soluble DC-SIGN-Fc as mentioned above. Thus infections of an individual with said pathogens can at least in part be prevented or treated by administering to said individual a

10 mannose carbohrate such as a glycoconjugate comprising at least two mannose residues in $\alpha 1,2$ linkage or a glycoconjugate comprising a fucose residue or a glycoconjugate comprising at least one end standing N-acetylglucosamine or a derivative or multimer thereof. In a preferred embodiment a ligand binding molecule of the invention is administered to said

15 individual. This embodiment can also be used to at least in part inhibit the binding of the pathogen to a C-type lectin expressing cell thereby at least in part inhibiting the contamination or spread of the pathogen in the body of a patient, as well as inhibiting DC maturation and cell adhesion, as is also described below.

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C-type lectins not only recognize carbohydrate profiles on pathogens but also interact with self glycoproteins to mediate cellular processes such as differentation and migration. A method of the invention can therefore also be used to interfere with the interaction with one or more self-glycoproteins and

25 thereby be used to at least in part inhibit the cellular processes that the mentioned C-type lectins are involved in. This can be done by providing the carbohydrates structures provided by the invention (i.e. mannose containing glycojugate such as a glycoconjugate comprising at least two mannose residues in $\alpha 1,2$ linkage or a glycoconjugate comprising a fucose residue or a

30 glycoconjugate comprising at least one end standing N-acetylglucosamine or

derivative or multimer thereof). Preferably, this is achieved using a ligand binding molecule of the invention. Thus in another preferred embodiment the method is used to at least in part inhibit binding of said C-type lectin to a self-glycoprotein preferably a receptor present on the outer membrane of a cell.

5 Preferably said receptor comprises ICAM-2, ICAM-3, CD11b, CD166 or CD66 or a functional part, derivative and/or analogue thereof. CD166 and CD66 are present on a subset of NK cells and granulocytes. Within the subset of CD66 variants the receptor preferably comprises CD66a and/or CD66e. Thus interaction of these cells with a cell comprising a mentioned C-type lectin can

10 be at least in part inhibited using a specific binding partner for a C-type lectin mentioned above. Thus interaction of preferably dendritic cells and said subset of NK cells and granulocytes can be interfered with. Through this interaction it is possible to modify an immune response. For instance, by at least in part inhibiting binding of a granulocyte to a dendritic cell, through providing a

15 binding molecule of the invention, the C-type lectin of the invention, it is possible to at least in part inhibit the mounting of an effective immune response against antigens carried by the granulocyte. On the other hand it is possible to augment or induce an effective antigen specific immune response by providing the antigen presenting cell with a ligand comprising said antigen

20 and a glycoconjugate comprising at least two mannose residues, a fucose residue and/or an end standing N-acetylglucosamine. Preferably, said C-type lectin comprises DC-SIGN. As specific binding partner of said C-type lectin a mannose containing glycoconjugate such as a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a

25 fucose residue or a glycoconjugate comprising at least one end standing N-acetylglucosamine or a derivative or multimer thereof, can be advantageously used. The cellular interaction of DC with granulocytes or NK cells is essential in the innate immune response. In particular granulocytes are involved early ingestion of pathogens and may attract and stimulate DC to participate in

30 pathogen recognition and presentation to T cells. In contrast NK cells are well

13

know to be involved in killing of infected cells. NK cells can kill or activate DC
that allow lysis of pathogen captured DC or maturation of pathogen captured
DC to enhance T cell stimulation and immune response. The cellular
interaction of granulocytes and NK cells with DC is to enhance immunity
5   against pathogens or eliminate pathogen infected DC.

In another aspect the invention provides a method for at least in
part inhibiting maturation or activation of an antigen presenting cell
comprising providing said antigen presenting cell with a DC-sign binding
10   glycoconjugate comprising at least two mannose residues, a fucose residue
and/or an N-acetylglucosamine residue. Using this technique it is possible to
for instance dampen an immune response. It is further possible to induce
antigen tolerance through this method, one can for instance provide antigen
together with the glycoconjugate. In case the antigen present cell is activated
15   through a toll-like receptor pathway the balance between the level of
activation/inhibition determines the maturation or activation of the antigen
presenting cell. When the TLR pathway is activated strongly whereas the C-
type lectin pathway is not, the balance is shifted toward activation of the
antigen presenting cell whereas in the reverse situation the balance is shifted
20   toward to tolerazing and inhibition of maturation or activation.

In another aspect the invention provides a method for modulating
the stimulating immune effect of a dendritic cell that is preferably activated
via a Toll-like receptor signaling pathway, said method comprising contacting
25   said dendritic cell with an isolated and/or recombinant specific C-type lectin
binding molecule. Preferably, said C-type lectin comprises DC-SIGN and said
specific C-type lectin binding compound comprises a glycoconjugate comprising
at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a
fucose residue or a glycoconjugate comprising at least one end standing N-
30   acetylglucosamine or a derivative, a combination or multimer thereof and

14

preferably, a glycoconjugate comprising at least two mannose residues in α1,2

linkage, preferably ManLAM. In combination with Toll-like receptor signaling

ManLAM binding to DC-SIGN on a dendritic cell induces the production of

significant amounts of IL-10 and reduces DC-activation/maturation. The

5   production of IL-10 and reduction of activation/maturation of DC lead to a

dampening of immune responses. Thus by providing a glycoconjugate

comprising at least two mannose residues in α1,2 linkage or an analogously

acting glycoconjugate to a dendritic cell, this cell can be induced to dampen the

immune response at least in its direct vicinity. Thus an active immune system

10   comprising a number of dendritic cells participating in the activity can be

dampened through by providing the dendritic cell with a glycoconjugate

comprising at least two mannose residues in α1,2 linkage or an analogously

acting glycoconjugate. This embodiment is useful in situations where an

individual is suffering from or at risk of suffering from an over-active immune

15   system. Preferably, said over-active immune system involves graft versus host

disease, host versus graft disease and the various auto-immune diseases.

Alternatively, an immune system dampened through the discussed

mechanism can be stimulated by providing dendritic cells with a specific C-

type lectin binding molecule capable of interfering with the binding of a

20   glycoconjugate comprising at least two mannose residues in α1,2 linkage or

analogously acting glycoconjugate. ManLAM, at least in amounts active

locally, is tolerated by individuals considering that active amounts of ManLAM

are secreted by mycobacterium infected cells in the body. Alternatively, the

dampened system can be stimulated by using a ligand binding molecule of the

25   invention, preferably a proteinaceous molecule such as a carbohydrate specific

antibody preferably specific for any mannose containing glycoconjugate such as

ManLAM or a glycoconjugate comprising a fucose residue or a derivative or

multimer thereof. Preferably, said antibody comprises a specificity for a

mannose residue in α1,2 linkage with another mannose. The experimental

30   part describes non-limiting examples of such antibodies. The ligand binding

molecule can also be a C-type lectin, preferably a soluble derivative thereof, comprising specificity for a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or a glycoconjugate comprising at least one end standing N-acetylglucosamine or a

5 derivative or multimer thereof. Another non-limiting example of such an interfering molecule is AraLAM. This embodiment is useful to combat (chronic) diseases wherein at least part of the phenotype of the disease is due to dampening of the immune system through the mentioned C-type lectin pathway. Preferred examples of such diseases are infections by a human

10 immunodeficiency virus, a *helicobacter*, a *neisseria meningitidis*, a *leishmania*, a *schistosoma*, a *klebsiella*, a probiotic lactobacillus, hepatitis C virus, a herpes simplex virus or an ebola virus

Human immunodeficiency virus binds to DC-SIGN through a glycoconjugate

15 having at least two mannose residues. *Helicobacter* and particularly *helicobacter pylori* binds to DC-SIGN through a glycoconjugate having a lewis^x antigen. *Neisseria meningitidis* binds to DC-SIGN through a glycoconjugate having N-acetylglucosamine residue. *Leishmania mexicana* binds to DC-SIGN through a glycoconjugate having at least two mannose residues. *Schistosoma*

20 *mansoni* binds to DC-SIGN through a glycoconjugate having a lewis antigen. *Klebsiella pneumonea* binds to DC-SIGN through a glycoconjugate having at least two mannose residues. Hepatitis C virus binds to DC-SIGN through a glycoconjugate having at least two mannose residues. Herpes simplex virus binds to DC-SIGN through a glycoconjugate having at least two mannose

25 residues. Ebola virus binds to DC-SIGN through a glycoconjugate having at least two mannose residues.
To alter the immune response in a body against the mentioned pathogens, a binding molecule of the invention thus preferably at least in part inhibits the binding of the respective glycoconjugates to the c-type lectin.

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Immature DC are highly efficient in antigen capture and processing, whereas
mature DC are specialized in the naïve T cell activation necessary for cellular
immune responses. Immature DC mature in response to specific 'danger'
signals such as bacterial components (LPS) or inflammatory cytokines (TNFa,
5   PGE2). In the present invention it was found that a glycoconjugate comprising
a mannose, a fucose residue or a derivative, a combination or multimer
thereof, does not induce DC maturation. In contrast to LPS that triggers
TLR4, no up-regulated expression of the activation markers CD80, CD83,
CD86 or HLA-DR was observed. However, when the maturation of immature
10   dendritic cells was studied in the situation when the cells are also activated
via a Toll-like receptor pathway, a strong inhibition of Toll-like receptor
induced maturation was observed, particularly when the dendritic cells were
exposed to ManLAM. Such inhibition thus dampens the response of Toll-like
receptor pathway stimulated dendritic cells and thereby the capacity of an
15   individual to cope with pathogens that are combated via activation of the Toll-
like receptor pathway. This dampening of DC-activation can be at least in part
prevented by at least in part inhibiting binding of particularly a glycoconjugate
comprising at least two mannose residues in α1,2 linkage, but also analogously
acting glycoconjugates to C-type lectins on the immature dendritic cells. The
20   invention thus provides a method for determining whether a compound is
capable of modulating an activation state of a dendritic cell comprising
providing said dendritic cell with a compound capable of specifically binding to
a c-type lectin and determining whether a Toll-like receptor signaling pathway
in said dendritic cell is modulated. Further provided is a method for
25   modulating the activity of a Toll-like receptor signaling pathway in a cell,
wherein said cell comprises a Toll-like receptor and a C-type lectin, said
method comprising contacting said cell with an isolated and/or recombinant C-
type lectin binding molecule. Preferably said C-type binding molecule
comprises a glycoconjugate comprising a mannose, a fucose residue or a
30   derivative, a combination or multimer thereof. Activity can be modulated

upward by at least in part preventing simultaneous stimulation of C-type lectin with a glycoconjugate comprising at least two mannose residues in α1,2 linkage or analogously acting compound. Activity is modulated downward by simultaneously providing said a glycoconjugate comprising at least two

5   mannose residues in α1,2 linkage or analogously acting compound. Analogously acting compounds comprise the same Toll-like receptor signaling interfering capacity as ManLAM in kind not necessarily in amount. A preferred analogously acting compound comprises a glycoconjugate comprising a fucose residue or a derivative or multimer thereof. A further level of control

10   is possible using C-type lectin binding compounds that at least in part interfere with the binding capacity of a glycoconjugate comprising at least two mannose residues in α1,2 linkage or an analogously acting compound. In this way activity of the Toll-like receptor pathway can be modulated upward (in the presence of the proper Toll-like receptor ligand) even in the presence of a

15   glycoconjugate comprising at least two mannose residues in α1,2 linkage or analogously acting compound. The latter feature is of importance in cases where the immune system has problems combating an infection via the Toll-like receptor pathway, particularly when said infection is accompanied with secretion of a glycoconjugate comprising at least two mannose residues in α1,2

20   linkage or analogously acting compounds, such as mycobacteria and in particular *M. tuberculosis* and *M. Bovis*. Thus the invention further provides a method for stimulating maturation of a dendritic cell that is contacted with a Toll-like receptor ligand and a glycoconjugate comprising at least two mannose residues in α1,2 linkage or analogously acting compound, said method

25   comprising providing said dendritic cell with a C-type lectin binding molecule capable of blocking the binding of said glycoconjugate to said C-type lectin. C-type lectin binding molecules capable of at least in part inhibiting the binding of a glycoconjugate comprising at least two mannose residues in α1,2 linkage or analogously acting compounds are for instance C-type lectin binding

30   antibody or a functional part, derivative and/or analogue thereof, with a

18

binding specificity that blocks or covers the ManLAM binding site on said C-type lectin. Preferable said C-type lectin comprises DC-SIGN. Thus in a preferred embodiment said antibody is a DC-SIGN specific antibody. A suitable example of such an antibody is AZN-D1, AZN-D2 or AZN-D3 or a

5   human or humanized analogue comprising the same binding specificity in kind not necessarily in amount. In a preferred embodiment the invention provides a method for stimulating maturation of a dendritic cell that is contacted with a Toll-like receptor ligand and a glycoconjugate comprising at least two mannose residues in α1,2 linkage or analogously acting compound, said method

10   comprising providing said dendritic cell with a ligand binding molecule of the invention, thereby at least in part preventing binding of said a glycoconjugate comprising at least two mannose residues in α1,2 linkage or analogously acting compound to the dendritic cell.

15   An important aspect of the invention is concerned with the use of a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or a glycoconjugate comprising at least one end standing N-acetylglucosamine or a derivative or multimer thereof, a ligand binding molecule of the invention and/or a C-type lectin

20   binding molecule of the invention for the preparation of a medicament. Such medicaments may be used for the treatment of an immune system associated disease or the treatment of an acquired disease. Preferably, said acquired disease comprises an infection with human immunodeficiency virus, a *helicobacter*, a *neisseria meningitidis*, a *leishmania*, a *schistosoma*, a *klebsiella*,

25   a probiotic lactobacillus, hepatitis C virus, a herpes simplex virus or an ebola virus.

           Considering the natural role of C-type lectins on antigen presenting cells it is within the scope of the present invention to stimulate immune

30   responses in an individual by providing antigen through a C-type lectin

19

receptor on the antigen presenting cell. This can of course be achieved when
the antigen comprises a a glycoconjugate comprising at least two mannose
residues in α1,2 linkage or glycoconjugate comprising a fucose residue or a
glycoconjugate comprising at least one end standing N-acetylglucosamine or
5   derivative or multimer thereof. In this embodiment it is preferred that
simultaneous activation of the Toll-like receptor signaling pathway is at least
in part prevented. Provided is thus a glycoconjugate comprising an antigen
and a glycoconjugate comprising at least two mannose residues in α1,2 linkage
and/or a fucose residue and/or at least one end standing N-acetylglucosamine
10   or a derivative or multimer thereof for use for the preparation of a vaccine. The
vaccine may be preventive or curative. The antigen may be derived from any
source as long as it is capable of being presented through major
histocompatibility complex I, complex II or C1b. In a preferred embodiment
said antigen comprises a tumor antigen. The presence of the mentioned
15   carbohydrates on tumor cells facilitates antigen capture by DC to enhance
antigen presentation and as a result immune activation. On the other hand,
the immune response against tumor antigen lacking the mentioned
carbohydrates can stimulated significantly by providing the antigen with one
or more of the mentioned carbohydrates thereby stimulating DC uptake and
20   thus the immune response against the antigen. This aspect is useful in the
preparation of vaccines.

DC-SIGN has been demonstrated to have a carbohydrate specificity for
mannose-containing carbohydrates, Lewis antigens and GlcNAc-containing
25   structures (Figure 33, 34, 63-66). We have demonstrated that the carbohydrate
specificity of L-SIGN is different since it does not recognize Lewis antigen
carbohydrate structures whereas it does recognize high mannose structures
(Figure 33 and 34). The murine homologue mSIGNR1 recognizes similar to
DC-SIGN high mannose and Lewis antigens, but has also specificity for

sialylated Lewis antigens in contrast to DC-SIGN and L-SIGN (Figure 33 and
34).

DC-SIGN is a C-type lectin in particular highly expressed on Dendritic cells.
Its function is to recognize antigen and process and present this very

5   efficiently into MHC class I and II molecules.

It has long been thought that DC-SIGN is a pathogen recognition receptor that
recognizes pathogens to activate the immune system, but as it seems now, the
long list of pathogens that target DC-SIGN all persist and escape immunity by
different mechanisms. We have now four examples of pathogens that target

10   DC-SIGN to survive in the host.

1. HIV-1 and HCV target DC-SIGN to ' hide' within DC and escape the
intracellular routing to the lysosomal compartment.

2. Secretion of ManLam by mycobacteria targets DC-SIGN to induce
downmodulation of TLR induced DC maturation, and induction of IL-10.

15   3. the human gastric pathogen *Helicobacter pylori* persists: Lipopolysaccharide
phase variants modulate the Th1/Th2 balance through interaction with the
dendritic cell lectin DC-SIGN.

4. Lactobacillus target DC-SIGN on DC and induce regulatory T cells, .
inhibition of recognition of Lactobacillus by DC-SIGN reduces the regulatory

20   state of T cells.

DC-SIGN is thus a receptor that 'normally' recognize self-antigen to tolerize
(Annual Review Immunology, 2004). We here demonstrate that targeting of
·DC-SIGN by lactobacilli can indeed induce regulatory T cells. Furthermore it
seems that DC-SIGN can also recognize the tumor antigen CEA (CD66e) in

25   particular Lewis X and Y. These tumor antigens can be secreted and suppress
DC activation in a similar way as ManLam of mycobacteria tuberculosis.
The interaction of DC with granulocytes is elucidated by the fact that DC- .
SIGN recognizes CD66a and CD11b on granulocytes. In particular Lewis X
antigen on these molecules is recognized. In that way DC-SIGN functions as a

30   cell adhesion receptor that mediates cellular interactions of granulocytes with

21

DC. This cellular adhesive function between granulocytes and dendritic cells
has never been reported but it is likely that the granulocyte DC interaction is
essential to form a bridge between the innate immune response and the
adaptive immune response in such a way that the granulocyte passes the

5   infectious agents to the DC mounting an adequate immune response. In
particular the granulocyte activates and matures the DC, inducing
upregulation of cytokines and costimulatory molecules which is necessary for
DC migration and initiation of the adaptive immune response. The interaction
of DC with granulocytes is also seen in vivo in the situation of Crohns disease.

10   A new pathogen that interacts with DC-SIGN on DC is *Neisseria Meningitidis*.
Strain variants demonstrate that in particular one strain mutant IgtB
interacts which contains endstending GlcNAc residue. Other experiments
(Figure 66) demonstrate the DC-SIGN also recognizes GlcNac.

15   In another embodiment the invention provides the use of ligand
binding molecule and/or a c-type lectin binding molecule of the invention for
the preparation of a vaccine. A dampening of the immune system due to the
effect of a glycoconjugate comprising at least two mannose residues in α1,2
linkage or analogously acting compounds is reduced by providing a

20   glycoconjugate or antibody capable of at least in part inhibiting the binding of
a glycoconjugate comprising at least two mannose residues in α1,2 linkage or
analogously acting compound to their C-type lectin receptor. The antibody
preferably comprises SMLDN1.1, SMFG4.1, 6H3 or SMLDN1.1. Thus antigen
present in said vaccine or provided separately is more effective in stimulating

25   or boosting an immune response in the presence of a glycoconjugate
comprising at least two mannose residues in α1,2 linkage or analogously
acting compound. This is particularly important in patients suffering from a
mycobacterial infection, such as but not limited to M. tuberculosis or *M. bovis.*
Preferably, said vaccine is used to stimulate an antigen specific immune

30   response in said individual. In a preferred embodiment the medicament or

22

vaccine is used for the treatment of an individual suffering from a cancer, an autoimmune disease or a transplantation related disease.

5   As used herein the term "antibody" refers to antibodies derived from humans or other animals. The antibody is preferably produced outside the body. The antibody can also be generated or selected using artificial systems such as phage display selection. An antibody having no natural counterpart is therefore well within the scope of the present invention. Antibodies as used

10   herein also include fragments thereof capable of binding to the same target, such as FAB fragments or even smaller parts. The antigen binding part of an antibody of the invention may also be grafted onto another type of molecule to provide that molecule with a binding specificity as provided for in the invention. Modification of the antibody to include human or humanized

15   versions thereof with the same binding specificity in kind not necessarily in amount are of course also in the scope of the invention. Also included are single chain fragments and variants thereof.

          As used herein the term proteinaceous molecule refers to a peptide,

20   a poly-peptide, protein and the like with or without modifications. Such modifications may be synthetic and/or provided for by a biological system. The latter including for instance post-translation modification such as glycosylation.

25   Where the invention is described for use in humans, the invention is also functional in other animals for instance farm-animals and pets. These medical and vaccine uses and methods of treatment are therefore also part of the invention.

30   Brief description of the drawings

Figure 1. Le^x – neoglycoconjugates bind with high affinity to DC-SIGN.

a. Schematic diagram of the covalent structures of the glycan chains of

synthetic glycoconjugates used in the DC-SIGN binding assay. b and c.

5   Glycoconjugates were coated and binding of recombinant DC-SIGN-Fc was

measured after incubation with peroxidase-labeled goat-anti-human Fc. Anti-

DC-SIGN monoclonal antibody AZN-D1 were used to block binding. c.

Titration of the glycoconjugates revealed that DC-SIGN binds with high

affinity to Le^x , Le^y, Le^a, Le^b, or LDNF –glycoconjugates and □1,3,□□1,6

10   mannotriose, sulfo-Lea, whereas it has a lower binding affinity for □-mannose

and □-L-fucose, and sialyl-Le^x.

Figure 2. Cellular DC-SIGN displays a binding specificity similar to that of

soluble DC-SIGN-Fc. Binding of DC-SIGN expressing K562 transfectants and

15   immature monocyte derived DC to glycoconjugate-coated fluorescent beads

was measured by FACscan analysis. Binding was inhibited by anti-DC-SIGN

monoclonal antibody AZN-D2. One representative experiments out of three is

shown. SD is less than 2 %. Inlay: Picture of Le^x-coupled fluorescent beads that

bind to DC-SIGN expressing DC.

20

Figure 3. DC-SIGN binds four novel pathogens

a. Pathogens that consist out of Le^x –rich Helicobacter pylori and Schistosoma

mansoni, and mannose-capped lipoarabinanomannan (manLAM) of

Mycobacterium tuberculosis and mannose-capped lipophosphoglycan of

25   Leishmania mexicana, Escherichia coli, Klebsiella pneumoniae, Pseudomonas

aeruginosa and Staphylococcus aureus were coated and binding of

recombinant DC-SIGN-Fc was measured as with peroxidase-labeled goat-anti-

human Fc.

b. DC-SIGN can bind LPS of Klebsiella Len1 that contains mannose cap, but

30   does not bind the LPS of Klebsiella Len 111 that lack a mannose cap

24

indicating that strains that contain the DC-SIGN binding carbohydrates
interact with DC-SIGN on Dendritic cells or DC-SIGN transfectants.

Figure 4. DC strongly interact with H. pylori LPS through DC-SIGN.

5   Biotinylated LPS (strains 11637 and M1019) was coated on streptavidin beads
and the adhesion to DC was determined with the fluorescent bead adhesion
assay. Specificity was determined by using antibodies against DC-SIGN and
the mannose receptor. Moreover, adhesion was determined in the presence of
mannan and EGTA.

10

Figure 5. DC-SIGN rapidly internalizes Le^x glycans to lysosomal
compartments. Synthetic biotinylated Le^x –PAA were added to DC-SIGN-
expressing K562 cells or immature monocyte derived DC for 30 min incubation
at 37°C. Cells were stained with anti-CD107a (LAMP-1, green) as a lysosomal

15 marker and avidine-Alexa 594 for localization of Le^x after permeabilization of
the cells. Co-localization of LAMP-1 and Le^x results in a yellow staining. .

Figure 6. DC-SIGN specifically binds ManLAM, a cell-wall component of M.
tuberculosis.

20 a)   DC-SIGN interacts with several mycobacteria strains. DC-SIGN-Fc
binding to mycobacteria ($5*10^5$ bacteria) was determined by a Fc-specific
ELISA. Specificity was determined by measuring binding in the presence of
blocking DC-SIGN-specific antibodies (AZN-D1 or AZN-D3) and mannan.
EGTA was used to determine the calcium-dependency of the DC-SIGN-Fc-

25 mediated binding. ICAM-3-Fc binding to mycobacteria was also measured to
exclude non-specific binding by the Fc domain. Standard deviation < 0.02
OD450. One representative experiment out of three is shown. Binding of
Mycobacterium Smegmatis that lacks the ManLAM structure does not interact
with DC-SIGN (data not shown).

b) The schematic structure of ManLAM. Lipoarabinomannan (LAM) of M.
tuberculosis consists of a glycosylphosphatidyl anchor (GPI) , an mannose-rich
oligosaccharide core and a branched arabinose polymer that ends in mannose-
caps (n=0-3). AraLAM has a similar structure but does not contain the

5   mannose-cap (ref).

c) The mannosylated-lipoarabinomannan ManLAM, in contrast to the non-
mannosylated AraLAM, is specifically bound by DC-SIGN. The anti-DC-SIGN
antibody AZN-D1 was used to determine specificity. The DC-SIGN-Fc binding
assay was performed as described in Figure 1a. Standard deviation < 0.02

10   OD450. One representative experiment out of three is shown.

d)   DC-SIGN does not interact with AraLAM. The DC-SIGN-Fc binding
assay was performed as described in Figure 1a. One representative experiment
out of three is shown.

15

Figure 7. Cellular DC-SIGN binds strongly to both viable mycobacteria and the
mycobacterial component ManLAM through its primary binding site.
a) K562-DC-SIGN transfectants express high levels of DC-SIGN but lack
expression of the other reported ManLAM receptors MR, CD11b and CD11c.

20   Transfectants were generated as previously described?. Open histograms
represent the isotype controls, and filled histograms indicate the specific
antibody staining.
b) DC-SIGN, expressed by K562 transfectants, binds strongly to intact M.
bovis BCG and the mycobacterial component ManLAM but not to AraLAM.

25   The adhesion of cells to the LAM glycans was determined using the fluorescent
bead adhesion assay. Binding to viable mycobacteria was determined by
measuring the binding of K562 transfectants to FITC-conjugated mycobacteria
(MOI 20) using flow cytometry. Specificity was determined by measuring
binding in the presence of blocking antibodies against DC-SIGN. Standard

30   deviation for the fluorescent bead adhesion assay and the mycobacteria

26

binding assay was < 5% and <2%, respectively. One representative experiment
out of three is shown.

c) The Val351 amino acid residue is not essential for the interaction of DC-
SIGN with *M. bovis* BCG and ManLAM, similar to HIV-1 gp120, whereas it is
5   essential for ICAM-3 binding. Binding to the V351G DC-SIGN mutant
expressed by K562 cells was measured as described in Figure 2. Specificity
was determined by measuring binding in the presence of blocking antibodies
against DC-SIGN, mannan or EGTA. Standard deviation <5% (fluorescent
bead adhesion assay) and <2% (mycobacteria binding assay). One
10   representative experiment out of three is shown.

d)Binding of DC-SIGN to differential components of ManLAM.
Neoglycoproteins Ara-Man, Ara-Man2, Ara-Man3 or Ara6 were coated on
fluorescent beads and added to DC-SIGN expressing cells. DC-SIGN preferably
binds Man2 and Man3 as demonstrated with the strong binding that can be
15   blocked by the addition of anti-DC-SIGN antibodies (AZN-D1).

Figure 8. DC-SIGN is an important receptor for both ManLAM and
mycobacteria on DC.

a) Immature DC express high levels of DC-SIGN and the other reported LAM
20   receptors MR, CD11b and CD11c. Open histograms represent isotype control
and filled histograms indicate specific antibody staining.

b) Immature DC bind strongly to ManLAM via DC-SIGN. Binding was
determined using the fluorescent bead adhesion assay. Specificity was
determined by measuring binding in the presence of mannan, EGTA or
25   blocking antibodies against DC-SIGN (AZN-D2), MR (Clone 19), CD11b (bear-
1) or CD11c (SHCL3). Standard deviation <5%. One representative experiment
out of three is shown.

c) DC-SIGN mediates capture of *M. bovis* BCG by immature DC. Binding was
determined by flow cytometry using FITC-conjugated mycobacteria. Specificity
30   was determined by measuring binding in the presence of antibodies against

27

DC-SIGN (AZN-D1, AZN-D2 and AZN-D3), MR (Clone 19), CD11b (bear-1) and CD11c (SHCL3). Binding was also measured in the presence of the C-type lectin inhibitors mannan and EGTA, whereas a known MR ligand, mannose-BSA, was used to determine the contribution of the MR receptor. Standard

5   deviation <2%. One representative experiment out of three is shown.

d) DC-SIGN mediates capture and internalization of *M. bovis* BCG by K562 cells. K562 transfectants were incubated with FITC-conjugated *M. bovis* BCG (MOI 20). Cells were washed, and surface FITC was quenched by exposure to trypan blue. Phagocytosis was determined by comparing the FITC labeling

10 before and after quenching using flow cytometry. Surface bound bacteria are represented by open bars, internalized by closed bars. Standard deviation <4%. One representative experiment out of three is shown.

e) Immature DC rapidly phagocytose mycobacteria through DC-SIGN. The internalization was determined as described in Figure 3b. Surface bound

15 bacteria are represented by open bars, internalized by closed bars. Standard deviation <5%. One representative experiment out of three is shown.

Figure 9. DC-SIGN mediates internalization of captured mycobacteria and ManLAM.

20 a) *M. bovis* BCG and ManLAM are internalized by DC-SIGN on immature DC and targeted to the lysosomes. The fate of captured mycobacteria was followed by analyzing immature DC pulsed with FITC-conjugated *M. bovis* BCG (MOI 20) for 2 hours using immuno-fluorescence microscopy (magnification 200x). ManLAM was followed by incubating DC with ManLAM (10 mg/ml) for 1 hour.

25 DC-SIGN, ManLAM and CD207a/Lamp-1 were stained with AZN-D1, F30.5 and H4A3, respectively. One representative experiment out of three is shown.

b) ManLAM induces down-regulation of DC-SIGN, but not of MR, CD11b and CD11c. Immature DC were incubated with 15 mg/ml of ManLAM or AraLAM for 18 hours, and then DC-SIGN expression was determined by flow cytometry.

30 One representative experiment out of three is shown.

28

Figure 10. Mycobacteria induce IL-10 production by DC through ManLAM and direct infection.

a) ManLAM induces IL-10 production of LPS-matured DC. Immature DC were incubated with 15 mg/ml of either ManLAM or AraLAM in the presence of LPS (10 ng/ml). The specificity was determined in the presence of blocking antibodies against DC-SIGN (AZN-D2; 20 µg/ml). Supernatants were harvested after 18 hours and the IL-10 production was measured by ELISA. Values are the means ± standard deviations of triplicate determinations. One representative experiment out of three is shown.

b) *M. bovis* BCG infection of immature DC induces IL-10 production. Immature DC were infected with *M. bovis* BCG (MOI 4), and the experiment was performed as described in Figure 5a. Values are the means ± standard deviations of triplicate determinations. One representative experiment out of three is shown.

Figure 11. ManLAM inhibits LPS-induced DC activation through DC-SIGN binding.

a) ManLAM does not induce activation of immature DC. Immature DC were incubated with ManLAM, AraLAM or LPS for 18 hours, and activation was determined by measuring the expression of CD80, CD86, CD83 and HLA-DR. Dotted lines represent isotype controls, the thin lines indicate expression levels of immature DC, and the thick line represents immature DC that have been treated with either LPS (10 ng/ml), ManLAM (15 µg/ml) or AraLAM (15 µg/ml). One representative experiment out of three is shown.

b) LPS-induced activation of DC is blocked by ManLAM. Immature DC were co-cultured with LPS alone, or together with either ManLAM or AraLAM for 18 hours. Dotted lines represent isotype controls. Thick lines, and the mean fluorescence values in the histograms, represent the expression levels after treatment with LPS alone, or in combination with either ManLAM or

AraLAM. Thin lines indicate the presence of antibodies against DC-SIGN throughout the incubation. One representative experiment out of three is shown.

5   Figure 12. *M. bovis* BCG induces maturation and ManLAM inhibits the induced DC activation through DC-SIGN binding.

a) *M. bovis* BCG induces maturation of immature DC. Immature DC were incubated with LPS or viable *M. bovis* BCG (MOI 4, 20 and 100) for 18 hours, and activation was determined by measuring the expression of CD80, CD86,

10 CD83 and HLA-DR. The mean fluorescence intensity is depicted. One representative experiment out of three is shown.

b) *M. bovis* BCG-induced activation of DC is blocked by ManLAM. Immature DC were infected with *M. bovis* BCG (MOI 4). Cells were pre-incubated with 15 µg/ml of either ManLAM or AraLAM and the expression of the markers was

15 measured after 18 hours as described in Figure 7a. Specificity was determined by pre-incubating cells with blocking antibodies against DC-SIGN (AZN-D2; 20 µg/ml). One representative experiment out of three is shown.

20 Figure 13. Structures of S. mansoni SEA carbohydrate antigens, monoclonal antibodies (Mabs) recognizing them, and reactivity of these Mabs with SEA. The reactivity of the MAbs was measured by ELISA, using SEA coated at a concentration of 1 µg/ml. The antibodies, incubated at a concentration of 1 µg/ml all showed a strong reaction with SEA.

25

Figure 14. Interaction of SEA with immature human DC.

a) Immature human DC were cultured from monocytes in the presence of GM-CSF and IL-4. The DC express next to high levels of DC-SIGN, the MR, CD83, CD86, CD80 and HLA-DR as determined by FACSscan analysis

b) DC-SIGN, expressed by immature DC, shows a similar binding to SEA as to
HIV-1 gp120, a previously defined ligand of DC-SIGN. The adhesion to both
SEA and HIV-1 gp120 was determined using the fluorescent bead adhesion
assay. Mannan and anti-DC-SIGN MAb AZN-D1 (20 µg/ml), but not anti-MR

5   MAb clone 19, block adhesion of SEA to the DC. One representative
experiment out of three is shown, using anti-LDN antibodies to couple SEA to
the fluorescent beads. Similar results are obtained using anti-LDN-DF
antibodies (not shown).

10   Figure 15. Binding of DC-SIGN to S. mansoni SEA.
DC-SIGN binds as strongly to SEA as to HIV-1 gp120, as was determined by
an ELISA based assay using soluble DC-SIGN-Fc. SEA were coated at a
concentration of 5 µg/ml, and HIV-1 gp120 at a concentration of 1 µg/ml.
Specificity was determined by measuring binding in the presence of the anti-

15   DC-SIGN blocking antibody AZN-D1 (20 µg/ml), or EDTA (5 mM).

Figure 16. DC-SIGN strongly binds SEA
Binding of soluble DC-SIGN-Fc to different concentrations SEA was measured
by an anti-IgG-Fc ELISA.

20

Figure 17. DC-SIGN binds a subfraction of SEA
SEA were separated by SDS-PAGE gelelectrophoresis on a 12.5%
polyacrylamide gel.
a) polyacrylamide gel with SEA (15 µg) silver-stained to detect all proteins

25   b) Western blot of polyacrylamide gel after SDS-PAGE of SEA (2 µg), using
soluble DC-SIGN-Fc to detect DC-SIGN binding glycoproteins.

Figure 18. DC-SIGN binds to fucosylated SEA and the α1,3-fucosylated
trisaccharide Le^x

a) SEA were defucosylated by mild acid treatment or α3,4-fucosidase. Binding of soluble DC-SIGN-Fc to defucosylated SEA (coated at 1 μg/ml) was measured by an anti-IgG-Fc ELISA. The degree of defucosylation, and the integrity of non-fucosylated glycans after the treatments was established using different
5   anti-glycan MAbs, and goat-anti-mouse IgM-peroxidase for detection.

b) Competitive inhibition of the binding of soluble DC-SIGNFc to SEA and HIVgp120 by anti-glycan mAbs in ELISA. Coated antigens were preincubated with anti-glycan mAbs before adding DC-SIGNFc. Binding of DC-SIGN Fc was measured by an anti-IgGFc ELISA. Antibodies usedpreferably SMLDN1.1 or
10   SMFG4.1 (anti-Le^x) or SMLDN1.1 (anti-LDNF) or anti-DC-SIGN (AZN-D1, D2, AZN-D3),

c) DC-SIGN stongly binds to neoglycoproteins carrying α1,3-fucosylated oligosaccharides Le^x and LDN-F but poorly to neoglycoconjugates carrying a single a-linked fucose or to LDN-DF. Neoglycoproteins were coated at a
15   concentration of 5 μg/ml. No binding was observed to neoglycoproteins carrying Galb1,4GlcNAc or GalNAcb1,4GlcNAc (LDN) (not shown).

Figure 19. Binding of SEA and Le^x to mutant DC-SIGN and L-SIGN

a) Amino acid sequence alignment of part of the CRDs of DC-SIGN
20   (AAK20997) with that of the highly homologous C-type lectin L-SIGN (AAK20998). The position of the E324A, E347Q and V351G mutations in DC-SIGN are indicated by an arrow.

b) Binding of SEA and Le^x-PAA coated beads to K562 transfectants expressing wild-type DC-SIGN and the E324A, E347Q and V351G DC-SIGN mutants,
25   was measured using the fluorescent bead adhesion assay. To detect SEA, fluorescent beads coupled to the LDN-DF MAb were used. One representative experiment out of two is shown.

c) SEA blocks binding of DC-SIGN, expressed by human immature DC, to ICAM-3 and HIV-1-gp120. In contrast, RNAseB does not block this binding.
30   The adhesion of both ICAM-3 and HIV-1 gp120 to DC was determined using

32

the fluorescent bead adhesion assay. Inhibitors were present at a

concentration of 20 μg/ml.

d) DC-SIGN, expressed by K562 transfectants, binds strongly to both SEA and

HIV-1 gp120, whereas L-SIGN, expressed by K562 transfectants only

5   recognizes HIV-1 gp120. The adhesion was determined using the fluorescent

beads adhesion assay. To detect SEA, both anti-LDN and anti-LDN-DF coated

fluorescent beads were used with similar results. One representative

experiment out of two is shown, using anti-LDN-DF fluorescent beads.

e) K562 transfectants efficiently express DC-SIGN, L-SIGN and the different

10   mutant DC-SIGNs, as determined by FACSscan analysis

Figure 20. Granulocytes strongly interact with DC-SIGN-coated beads, partly

through Le^x-containing ligands.

A.   Both mannose as well as Lewis-X epitopes are highly expressed on

15   glycans expressed by granulocytes

B.   DC-SIGN binds strongly to freshly isolated granulocytes. Freshly

isolated granulocytes are incubated with DC-SIGN-Fc-coated fluorescent

beads. The adhesion is determined by the fluorescent bead adhesion assay.

Specificity is determined by measuring the adhesion in the presence of

20   mannan, EGTA and antibodies against DC-SIGN (AZN-D1,D2 or D3), b2

integrins (NKI-L19) and Le^x (6H3)

Figure 21. DC-SIGN-Fc has a high affinity for granulocytes.

The interaction of DC-SIGN with granulocytes is further investigated by

25   titration of DC-SIGN-Fc and determining the binding by measuring the bound

DC-SIGN-Fc using FITC-conjugated Goat-anti-human Fc antibodies. The

interaction is blocked by antibodies against DC-SIGN (AZN-D1), and mannan

and EGTA (A) and by anti-Le^x antibodies (3H3, B).

33

Figure 22. A 66kDa protein (CD66) is the high affinity ligand of DC-SIGN on
granulocytes.

A. Granulocytes are surface labelled with biotine. The cell lysate is incubated
over night at 4°C with Protein A beads coated with DC-SIGN-Fc or control

5   antibodies against ICAM-2, ICAM-3 and LFA-1. The beads are washed and the
immuno-precipitated product is analysed on SDS-PAGE and visualised by
autoradiograph. In particular a protein size 66kD is immuno-precipitated. B.
Granulocytes highly express the Le^x containing CD66acd and CD66d antigen,
do not express the DC-SIGN ligand ICAM-2 and express ICAM-3. C. Immuno-

10   precipitation of CD66acd demonstrates strong binding activity for DC-SIGNFc.
Anti-CD66acd mAbs coated on an ELISA plate were incubated with a lysate of
granulocytes. After washing off non-specific proteins DC-SIGNFc was
incubated to demonstrate high affinity ligands. DC-SIGNFc does not recognize
ICAM-3 from granulocytes demonstrating that on granulocytes not ICAM-3

15   but CD66acd is the high affinity ligand for DC-SIGN. Binding of CD66acd to
DC-SIGNFc is completely inhibited by removal of cations by EDTA.

Figure 23. Cellular DC-SIGN strongly binds to the granulocyte-specific protein
CD66a.

20   293T cells are transfected with pIG-CD66a-Fc, pIG-ICAM-3-Fc and pIG
(mock). Supernatants are harvested and beads are coated with the Fc chimeras
(CD66a-Fc, ICAM-3-Fc and mock). Immature DC and DC-SIGN-transfected
K562 cells are incubated with beads and the adhesion is determined in the
presence of antibodies against DC-SIGN and β2 integrins, and mannan and

25   EGTA.

Figure 24. NK cells specifically interact with DC-SIGN-Fc.
The binding of DC-SIGN-Fc to peripheral blood lymphocyte (PBL) subsets is
determined by flow cytometry in the presence of antibodies against DC-SIGN.

34

PBL subsets are distinguished by staining with CD3 and CD56, and adhesion is determined by triple staining with DC-SIGN-Fc.

Figure 25. NK cells specifically interact with DC-SIGN-Fc in a concentration-
5   dependent manner. The binding of DC-SIGN-Fc to peripheral blood lymphocyte (PBL) subsets is determined by flow cytometry in the presence of antibodies against DC-SIGN. NK cells are detected by triple staining with CD3, CD56 and DC-SIGN-Fc. The CD56 dim and bright cells do not differ in ICAM-2 or ICAM-3 expression demonstrating that differential glycosylation is
10 the result of the high affinity binding of DC-SIGNFc to CD56 dim NK cells.

Figure 26. CD56^{dim}CD16^{+} NK subset specifically interacts with DC-SIGN-Fc. The binding of DC-SIGN-Fc to NK subsets is determined by flow cytometry in the presence of antibodies against DC-SIGN. NK subsets are distinguished by
15 staining with CD16 and CD56, and the adhesion is determined by triple staining with DC-SIGN-Fc.

Figure 27. A 166kDa protein and ICAM-2 is recognized by DC-SIGN-Fc on NK cells.
20 NK cells are surface labelled with radioactive iodine. The cell lysate is incubated over night at 4°C with Protein A beads coated with DC-SIGN-Fc or control antibodies against ICAM-2, ICAM-3 and LFA-1. The beads are washed and the immuno-precipitated product is analysed on SDS-PAGE and visualised by autoradiography.

25

Figure 28. Maturation of DC by NK cells is mediated by LFA-1.
Immature DC are co-cultured with NK cells for 24 hours in the presence of antibodies against DC-SIGN (AZN-D1) and LFA-1 (NKI-L19). The maturation is determined by measuring the expression of CD80, CD83, CD86 and HLA-

DR. with NK cells O/N at 37°C. As a control, DC are matured with LPS (10
ng/ml).

Figure 29. NK-mediated lysis of immature DC is dependent on DC-SIGN.
Chromium^{52}-labeled immature or LPS-matured DC are co-cultured with IL-2-
activated or non-activated NK cells for 4 hours in the presence of antibodies
against DC-SIGN (AZN-D1) and LFA-1 (NKI-L19). The lysis is determined by
measuring the Cr^{52} release.

Figure 30. NK-mediated lysis of K562 transfectants is enhanced by DC-SIGN.
Chromium^{52}-labeled K562 cells are co-cultured with IL-2-activated or non-
activated NK cells for 4 hours in the presence of antibodies against DC-SIGN.
The lysis is determined by measuring the Cr^{52} release.

Figure 31. DC-SIGN has a high affinity for HSV-1 and HSV-2.
HSV is coated in ELISA plates and DC-SIGN-Fc binding is determined in the
presence of antibodies against DC-SIGN.

Figure 32. DC-SIGN strongly binds HSV envelope glycoprotein gB.
A.DC-SIGN transfectants are incubated with HSV envelope glycoprotein-
coated beads and DC-SIGN binding is determined in the presence of antibodies
against DC-SIGN.
B L-SIGN strongly binds HSV envelope glycoprotein gB.
L-SIGN transfectants are incubated with HCV envelope glycoprotein-coated
beads and DC-SIGN binding is determined in the presence of antibodies
against DC-SIGN.

Figure 33A. mSIGNR1 binds mannose-containing carbohydrates, similar to
DC-SIGN and L-SIGN. K562 cells expressing human DC-SIGN, human L-

SIGN or murine mSIGNR1 bind in a similar extent mannose-containing
carbohydrates using the fluorescent bead adhesion assay.

Figure 33B. DC-SIGN, L-SIGN and mSIGNR1 bind differently to Lewis
5   antigens. Transfectants expressing human DC-SIGN human L-SIGN or
murine mSIGNR1 bind in a similar extent Lewis antigen coupled to
fluorescent beads. DC-SIGN and mSIGNR1, in contrast to L-SIGN, recognize
Lewis X antigen. Murine mSIGNR1 interacts with sialyl Lewis X in contrast
to human DC-SIGN and L-SIGN.
10

Figure 33C Carbohydrate specificity of DC-SIGN, L-SIGN and mSIGNR1 is
different. Lewis X antigen is not recognized by L-SIGN, while human DC-
SIGN and mSIGNR1 do. mSIGNR1 recognizes sialyl Lewis X in contrast to
DC-SIGN and L-SIGN. Both human DC-SIGN and murine mSIGN1 bind sialyl
15   Lewis A, in contrast to L-SIGN.

Figure 34. DC-SIGN, L-SIGN and mSIGNR1 bind to yeast-derived mannan,
Hepatitis C virus E1/E2, HIV-1 gp120 and mycobacterial ManLAM. The
pathogenic ligands have been coated on fluorescent beads and the adhesion
20   was measured using the fluorescent bead adhesion assay with K562
transfectants. Gp120 binding to DC-SIGN, L-SIGN and mSIGNR1 was
inhibited using the yeast-derived carbohydrate mannan, demonstrating that
these C-type lectins also bind yeast-derived mannan.

25

Figure 35. DC-SIGN specifically binds HCV envelope proteins.
A. DC-SIGN binds both yeast Hansenula and mammalian cell produced HCV
envelope proteins. DC-SIGN interaction with HCV envelope proteins E1 and
E2 (2 mg/ml) produced by the yeast Hansenula or mammalian cells infected
30   with recombinant vaccinia virus was tested in a Fc-based ELISA. Specificity of

the binding was confirmed using the DC-SIGN specific blocking antibody AZN-

D1 (50 mg/ml), mannan (100 mg/ml) or the calcium chelator EGTA (5 mM).

B. DC-SIGN interaction with HCV E1 and E2 is concentration dependent.

Mammalian cell produced E1 and E2 were titrated (0-16 or 14 mM resp.) and

5   DC-SIGN-Fc binding was tested as described above. AZN-D1 (50 mg/ml) was

used to specifically block the interaction.

C. DC-SIGN has a similar affinity for E1, E2 and gp120. Mammalian cell

produced E1 and E2 (10 nM) and gp120 (2 nM) were coated and DC-SIGN-Fc

binding was tested as described above. Mannan was titrated (0-1000 mg/ml) to

10   block the interaction. Binding was expressed as percentage of the binding

without block.

D. DC-SIGN binding to E1, E2 and gp120 is equally dependent on calcium.

Mammalian cell produced E1 and E2 (10 nM) and gp120 (2 nM) were coated

and DC-SIGN-Fc binding was tested as described above in the presence of a

15   varying of calcium (0-5 mM). Binding was expressed as percentage of binding

with 5 mM calcium.

Figure 36. HCV envelope proteins are bound by cellular DC-SIGN and L-

SIGN. A K562 transfectants express similar levels of DC-SIGN and L-SIGN. B

20   Cellular DC-SIGN and L-SIGN bind to both envelope glycoproteins E1 and E2.

K562 cells transfected with DC-SIGN and L-SIGN were used to measure

binding to HCV envelope protein with the fluorescent-coated bead adhesion

assay. C The DC-SIGN Val351 mutant binds both HCV envelope proteins E1

and E2. DC-SIGN V351G binding to glycoproteins E1 and E2 coated beads was

25   investigated and the specificity was determined by blocking the interaction

with the DC-SIGN specific antibody AZN-D2 or the calcium-ion chelator

EGTA. D. Mutational analysis demonstrate that similar aminoacids within the

CRD region of DC-SIGN are involved in the recognition of HCV and HIV-1 and

that those are also involved in ICAM-3 binding.

30

Figure 37. Internalization pathway of DC-SIGN is dependent on the cellular background. A DC-SIGN and L-SIGN expressed by K562 and THP-1 transfectants bind HCV VLPs similarly. Binding of HCV E1/E2 VLP to K562 and THP-1 transfectants with both DC-SIGN and L-SIGN was measured using the fluorescent bead adhesion assay. B-E DC-SIGN-bound HCV is targeted to the early endosomes (transferrin+) in THP-1 cells, in contrast to the lysosomal (LAMP-1+) targeting in K562 cells. K-562 (B and C) and THP-1 cells (D and E) expressing DC-SIGN or L-SIGN were incubated overnight with HCV VLPs. HCV was detected using a human anti-HCV antibody and a fluorescent (FITC)-labeled secondary antibody. Intracellular targeting was determined by staining the endosomal compartments with a mouse antibody to the lysosomal/late endosomal specific LAMP-1 and fluorescent(Alexa Fluor 594)-labeled secondary antibody (B, D), or by co-incubating the cells for 15 minutes with Alexa Fluor 594-labeled transferrin that is specifically transported to the early endosomes and functions as a marker (C, E). Cells were analyzed by fluorescence microscopy.

Figure 38. Dendritic cells strongly bind to HCV E1 and E2 through DC-SIGN. A Immature DCs express high levels of DC-SIGN. Monocyte-derived dendritic cells were isolated as described in materials and methods. Expression of DC-SIGN was measured by FACS-staining with the DC-SIGN specific antibody AZN-D2. B, C Immature DCs and mature DCs bind strongly to HCV envelope proteins E1 and E2, and mixed HCV E1/E2 VLPs via DC-SIGN. Immature (B) and LPS-matured DCs (C) binding to HCV envelope proteins was determined by a fluorescent bead adhesion assay. Specificity was determined by anti-DC-SIGN antibody AZN-D2 (50 mg/ml), mannan (100 mg/ml), EGTA (10 mM), and anti-mannose receptor antibody (clone 19) (50 mg/ml).

Figure 39. DC-SIGN on iDC targets HCV VLPs to early endosomes. A Immature dendritic cells were incubated with HCV VLPs (30 mg/ml) for 4

hours or overnight. HCV was detected with a human anti-HCV antibody and a
FITC-labeled secondary antibody. Intracellular targeting was determined by
staining the endosomal compartments with a mouse antibody to the
lysosomal/late endosomal specific LAMP-1 or the early endosome specific
5   marker EEA-1 and an Alexa Fluor 594 labeled secondary antibody, or by co-
incubating the cells for 15 minutes with Alexa Fluor 594 labeled transferrin
which is specifically transported to the early endosomes. B Immature dendritic
cells were incubated with HCV VLPs (30 mg/ml) for 4 hours or overnight at 37
or 4°C. HCV was detected as described for figure 5A. Localization of DC-SIGN
10   was determined with the DC-SIGN specific antibody AZN-D2 and an Alexa
Fluor 594 labeled secondary antibody.

Figure 40 DC-SIGN on iDCs targets its ligand Lewis X antigen to late
endosomes/lysosomes. Immature dendritic cells were incubated with Lewis X
15   (10 mg/ml) for 4 hours. Intracellular targeting was determined by staining the
endosomal compartments with a mouse antibody to the lysosomal/late   .
endosomal specific LAMP-1 or by co-incubating the cells for 15 minutes with
Alexa Fluor 594 labeled transferrin which is specifically transported to the
early endosomes. Cells were analyzed by fluorescence microscopy.

20
Figure 41. HCV interacts with L-SIGN expressing human liver sinusoidal
endothelial cells (LSECs) in situ. (A) L-SIGN is expressed by human LSECs,
as was determined by staining of liver tissue with an L-SIGN specific antibody.
(B) Binding of HCV VLPs by liver tissue was determined by incubating liver
25   sections with HCV VLPs for 2 hours at 37°C. HCV binding was detected using
a mouse anti-HCV antibody and an Alexa Fluor 594 labeled secondary
antibody. (C) HCV VLP binding to LSECs was blocked by the calcium chelator
EGTA.

Figure 42. Lewis blood group antigens and some of their related structures
bind to DC-SIGN. (a) Lewis blood group antigens expressed by H. pylori .
(b-c) Carbohydrates, representing blood group antigens or their substructures,
conjugated to polyacrylamide (b), or ceramide (c), were coated and binding of

5   recombinant DC-SIGN-Fc was measured using the DC-SIGN-Fc ELISA.

Figure 43. Binding of H. pylori is dependent on Lewis antigen expression.
(a-c) H. pylori α3-fucosyltransferase mutants (a), strains (b) and phase
variants (c) were coated and binding of recombinant DC-SIGN-Fc was

10 measured after incubation with peroxidase-labeled goat-anti-human Fc. H.
pylori were coated and incubated with antibodies specific for Lewis antigens as
indicated and their serotype was determined after incubation with peroxidase-
labeled anti-mouse immunoglobulins.

15 Figure 44. LPS phase variation in H. pylori occurs in vivo.
(a) Lex/y positive phase variants of J223 were detected by colonyblotting with
mAbs specific for the indicated Lewis antigens, after short time culturing
directly from the biopsy, followed by one single passage in fluid phase and
distribution over solid media. (b) C-tract sequencing was performed to

20 determine the "on" and "off"-status of genes futA (HP0379) and futB (HP0651).
The J223.3 futB mutant was generated by natural transformation with
construct containing a chloramphenicol resistance marker cassette inserted in
gene HP0651, and serotyped as indicated for J223. (c) Consequences of the C-
tract length for functional expression of futA and futB.

25

Figure 45. DC-SIGN is expressed on gastric DCs and is the major receptor for
Le positive H. pylori.
(a) A tissue section of stomach was fixed and stained with anti-DC-SIGN
antibodies. Original magnification x 20. Arrows indicate DC-SIGN positive

30 DC-like cells in the lamina propria. (b-d) Monocyte derived DCs (b), monocyte-

derived DCs and RAW macrophages (c) or K-562 cells transfected with DC-
SIGN (d) were incubated with FITC labelled H.pylori J223.3 or J223.8 and
binding was analyzed using flowcytometry. In b and d, cells were preincubated
with anti-DC-SIGN antibodies, mannan, EDTA, or anti-mannose receptor

5   (MR) antibodies.

Figure 46. Binding of H. pylori to induces DC-SIGN-dependent increase of
cytokines IL-10 and IL-12 production, but no changes in IL12p70.
(a) DC were incubated with H. pylori J223.3 or J223.8 at an multiplicity of

10   infection (M.O.I.) of 20 in the presence or absence of anti-DC-SIGN antibodies
for 1 hr, washed and cultured for 20 hrs. Supernatant was harvested and the
amount of IL-10 and IL-12p40 was analyzed by ELISA.
(b) Upon coculture of DC with H. pylori J223.3 or J223.8, cells were incubated
with CD40L-transfected J558 fibroblasts in the absence or presence of IFN-γ

15   for 24 hrs. Supernatant was harvested and the amount of IL-12p70 was
analyzed by ELISA.

Figure 47. Binding of H. pylori to DC-SIGN induces skewing of naïve T cells to
Th2.

20   After preincubation with anti-DC-SIGN antibodies, DC were incubated with H.
pylori J223.3 or J223.8 at an M.O.I. of 10 for 48 hrs, washed and subsequently
cocultured with highly purified CD45RA+CD4+ T cells. Quiescent T cells were
re-stimulated with PMA and ionomycin and IL-4 and IFN-γ was analyzed on a
single-cell basis by intracellular flowcytometry.

25

Figure 48: Lactobacilli induce partial DC maturation.
Immature DC (iDC) were generated as described A) Maturation was induced
by addition of LPS (100 ng/ml), 107 bacteria, LPS plus MF (rIL-1b (25 ng/ml)
and rTNFa (50 ng/ml)), or MF plus 1.107 bacteria. After 48 h, mature DC

30   (mDC) were harvested, washed and (A) surface expression of CD86, HLA-DR

and CD83 was analyzed by flowcytometry. (B) Mature DC (2.104 cells/well)
were stimulated with mouse CD40L-expressing mouse fibroblast cells (J558
cells, 2x104 cells/well) to induce the production of IL-12, IL-10 and IL-6. After
24h, supernatants were collected and IL-12p70, IL-10 and IL-6 production

5   were measured by ELISA.

Figure 49: L. reuteri and L. casei induce regulatory T cell development by
modulation of DC function.

Generation of iDC and maturation conditions are described in the legend to

10   figure 16. (A) Mature DC (5x103 cell/well) were cocultured with naive helper T
cells (Th cells) (2x104 cells/well) and superantigen SEB (10 pg/ml). After 12
days, IFN-g and IL-4 productions per cell were analyzed by intracellular
FACS-staining following a 6h PMA/ionomycin stimulation, the last 5h in the
presence of Brefeldin A. (B) Mature DC (2,5x103 cell/well) were cocultured

15   with naive Th cells (5x104 cells/well). Cell proliferation was assessed by the
incorporation of radioactively-labeled [3H]-TdR after a pulse with 13 KBq/well
during the last 16 h of 6-day culture after stimulation, as measured by liquid
scintillation spectroscopy. (C) Naive Th cells were stimulated as described in
part A. After 12 days, the test cells were fluorescently-labeled with PKH, and

20   stimulated with suboptimal concentrations of anti-CD3 (1:5000) and anti-
CD28 (1:2000). After overnight incubation, fluorescent(CFSE)-labeled target T
cells (peripheral CD4+ T cells), were added in a 1:1 ratio (2,5x104 each). After
5 days the PKH and CFSE staining of the cells were analyzed by
flowcytometry. The gray shade CFSE profile represents the test condition

25   (bacteria: 105), whereas the overlay indicates the proliferation in the presence
of control test cells (MF/LPS). The figure is a representative out of 7
independent experiments. (D) The mean fluorescence intensity (MFI) of the
CFSE-labeled target cells cocultured in the presence of control test cells
(MF/LPS) was set at 100%, representing the maximal proliferation. The MFI of

30   the target T cells cocultured with other test cells was compared to this value,

calculating the relative proliferation. The results are expressed as the mean percentages ± SEM from 4-7 independent experiments (Cordycepin: 25 – 12,5 – 6,25 µg/ml). Data were analyzed for statistical significance using ANOVA followed by Bonferroni's multiple comparison test. * P<0,05, ** P< 0,01.

5

Figure 50:No TLR activity by Lactobacilli.

(A) Stable transfected HEK 293 cells with either TLR2 or TLR4 were seeded at 0,2x106 cells/well and stimulated with 107 bacteria/ml or TNFa . After 24 hours, supernatant was taken and IL-8 content was analyzed by ELISA. (B)

10 HEK 293 cells were transiently transfected with human TLR1, TLR2, TLR3, TLR4 + MD2, TLR6, TLR7 and TLR9, together with an ELAM-luciferase reporter construct and were stimulated with 107 bacteria or TNFa. Luciferase activity was determined 6h after stimulation.

15 Figure 51: L. reuteri and L. casei ligate DC-SIGN on moDC.

(A). Immature DC were incubated with FITC-labeled bacteria. After washing, the cells were analyzed by flow cytometry. (B) Similar protocol, as described in the part A, was used to analyze bacteria binding to DC-SIGN-transfected K562 or untransfected K562 cells. Preincubations were performed with either TSM +

20 0,5% BSA, EDTA or anti-DC-SIGN (AZN-D1 and AZN-D2, 20 µg/ml). (C) DC-binding was performed as described in part A. Preincubations were performed with either TSM + 0,5% BSA, anti-DC-SIGN (AZN-D1 and AZN-D2, 20 µg/ml), anti-MR, Mannan or methylglucoside .

25 Figure 52: Blocking of DC-SIGN interaction with L. reuteri and L. casei abrogates the priming for regulatory T cell development.

Generation of iDC is described in the legend to figure 1. Immature DC were preincubated (30 min, 37 °C) with either isotype control Ab, anti-DC-SIGN (AZN-D1; 20 µg/ml) or Mannan . Thereafter maturation was conducted as

described in the legend to figure 16. Induction of suppressor cell activity was analyzed as described in the legend to figure 17.

Figure 53. Polymorphic Mononuclear Neutrophisl (PMN) express the DC-SIGN
5   ligand Lewisx and bind with high affinity to recombinant DC-SIGN. A. Polyacrylamide glycoconjugates of Lewisx and sialylated Lewisx were coated and adhesion of DC-SIGN-Fc was measured with peroxidase labeled goat anti-human Fc in DC-SIGN-Fc ELISA. B. Lewisx expression was determined on PMN labeled for CD16 to discriminate between CD16+ neutrophils and CD16-
10 eosinophils. C. The adhesion of beads coated with ICAM-3-Fc or DC-SIGN-Fc to PMN was assessed using flow cytometry. Specificity of adhesion was obtained using blocking anti-DC-SIGN antibodies (AZN-D1, 20 mg/ml), mannan (50 mg/ml), or EGTA (10 mM). D. Titration of DC-SIGN-Fc to PMN reveals high affinity binding of DC-SIGN to PMN. E. Binding of optimal
15 concentration of DC-SIGN-Fc (10 mg/ml) to PMN labeled for the neutrophil marker CD16. To assess specificity blocking anti-DC-SIGN antibodies (AZN-D1, 50 mg/ml) were used.

Figure 54. CEACAM1 expressed on PMN is a ligand of DC-SIGN and binds
20 through its Lewisx moieties. A. Whole PMN lysate was analysed on a 7% polyacrylamide gel for 1.5 hr at 120 V, transferred onto nitrocellulose blot, and stained with DC-SIGN-Fc, anti-CEACAM1 (anti-CD66) or anti-Lewisx antibodies (3 mg/ml) and alkaline phosphatase conjugated secondary antibodies. B. Protein A (protA) beads coated with DC-SIGN-Fc were used to
25 immunoprecipitate DC-SIGN ligands from surface-biotinylated PMN. Immunoprecipitates were run on gel, transferred onto blot, and stained with peroxidase-conjugated streptavidin. C. CEACAM1 immunoprecipitated from PMN using protein G (protG) beads coated with anti-CEACAM1 antibodies was stained on blot using DC-SIGN-Fc and alkaline phosphatase conjugated
30 goat anti-human antibodies. D. Incubation with the enzyme a1-3,4 fucosidase

(5 mU/ml) for 3 days at 37°C removes Lewisx epitope on PMN-derived
CEACAM1. E. Using an ELISA-based system the adhesion of DC-SIGN-Fc to
PMN CEACAM1 was measured and compared to that after treatment with al-
3,4 fucosidase. F. Antibodies directed against Lewisx (20 mg/ml) block the

5   adhesion of DC-SIGN-Fc to CEACAM1 captured from PMN using anti-
CEACAM1 antibody in adhesion ELISA.

Figure 55. Cellular DC-SIGN expressed on K562 transfectants and immature
DC binds native CEACAM1 from PMN. A. Fluorescent beads coated with PMN

10   CEACAM1 were incubated with K562 transfectants expressing wildtype DC-
SIGN or mutant DC-SIGN displaying a single amino acid substitution in the
C-type lectin domain crucial for ligand binding. Bead adhesion was measured
using FACS analysis. B. Adhesion of CEACAM1 coated beads to immature DC
was determined. Specificity of adhesion was assessed using blocking anti-DC-

15   SIGN antibodies (20 mg/ml), mannan (50 mg/ml), or EGTA (10 mM).

Figure 56. DC-SIGN is involved in clustering of DC and PMN. A. CFSE-
labeled PMN (green) were incubated with fluorescent HE-labeled K562, K562-
DC-SIGN, and immature DC (red) for 15 min at 37°C. Anti-DC-SIGN

20   antibodies (AZN-D1 and AZN-D2, 10 mg/ml) were used to determine
involvement of DC-SIGN. Cell clustering was visualized using fluorescence
microscopy and representative pictures were taken. B. Using FACS analysis
cell clustering was followed in time by scoring the percentage of K562 or K562-
DC-SIGN that have bound PMN. C. Adhesion of PMN to immature DC was

25   quantified by flow cytometry.

Figure 57. Localization of PMN and DC in colonic mucosa of patients with
Crohn's disease. A. Slides of inflammatory intestinal tissue of patients with
Crohn's disease were double stained for the PMN markers Lewisx (6H3, green
fluorescence) and CEACAM1 (CLB-gran/10, blue fluorescence), B. for

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CEACAM1 (CLB-gran/10, green fluorescence) and the DC marker DC-SIGN
(CSRD, red fluorescence), or C. for Lewisx (6H3, green fluorescence) and DC-
SIGN (CSRD, red fluorescence). The anti-CEACAM1 antibody produces
background staining in crypts of the colon, probably due to crossreaction with

5   CEA that is expressed on colonic epithelial cells.   ,

Figure 58. PMN activate immature DCs through DC-SIGN binding. A.
Maturation of DC by resting o PMN was determined measuring the expression
of the DC maturation marker CD86. The maturation can be inhibited with

10   anti-DC-SIGN antibodies (AZN-D1). The DC maturation by PMNs was
compared with the maturation of DCs with LPS or E.coli. B. Cytokine
production of IL-12 and TNFa was analysed upon PMN DC interaction, and is
dependent on DC-SIGN binding since antibodies against DC-SIGN block the
induction of cytokines.

15

Figure 59. DC-SIGN binds both Lewis X-expressing CD11band CD66a-d on
PMN.
A Capture of CD11b and CD66acd from PMN lysate using specific antibodies
revealed the presence of Lewis X antigens on these cell membrane molecules

20   B Capture of CD11b and CD66acd from PMN lysate and coupling to
fluorescents beads revealed that in particular PMN –derived CD11b and
CD66acd interacted strongly with DC-SIGN expressing transfectants
indicating that DC-SIGN binds PMN derived CD11b and CD66acd.
C Captured CD11b/CD18 and CD66 out of PMN lysate demonstrates high

25   affinity binding with DC-SIGNFc as demonstrated in DC-SIGN Fc ELISA.
D. Immunoblotting studies demonstrate that DC-SIGNFc specifically
interacts with CD66acd and CD11b from PMN when immunoprecipitated (IP)
using anti-CD66acd (lane 9) and CD11b mAbs (lane 10) out of a PMN lysate.
Cell-surface proteins are biotinylated and PMNs are lysed. Lane 1. negative

30   control using ICAM-3 Fc to capture by IP proteins. Detection is peroxidase-

labeled streptavidin. Lane 2. DC-SIGN-Fc is used to capture ligands
biotinylated ligands from lysate; two protein bands are visible, the upper band
consists of CD11b and CD66a, whereas the lower consists of CD18. Lane 3.
Negative control; no bands are immune precipitated with anti-DC-SIGN
5   antibodies. Lane 4, IP with antibodies against CD66acd; a faint band is visible
containing CD66a. Lane 5, IP of CD11b; two bands are visible; the upper band
is CD11c and the lower band is CD18m, which is co-immuneprecipitated with
CD11c. Lane 6. IP with DC-SIGN ligands and detection with anti-CD66acd;
CD66a is captured by DC-SIGN Fc. Lane 7. IP with DC-SIGN ligands and
10   detection with anti-CD11b; CD11b is captured by DC-SIGN Fc. Lane 8.
Negative control, IP with anti-DC-SIGN antibodies and detection with DC-
SIGN-Fc. Lane 9. IP with anti-CD66acd and detection with DC-SIGN Fc;
CD66a is bound by DC-SIGN on blot. Lane 10. IP with anti-CD11a and
detection with DC-SIGN Fc; CD11a is bound by DC-SIGN on blot.

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Figure 60
DC-SIGN binds Lewis X structures on CD11b form PMN.
Immunoprecipitation (IP) studies revealed that binding of DC-SIGN-Fc to
CD11b is abrogated when CD11b is treated with a-1, 3,4 Fucosidase that
20   removes the fucose residue present in Lewis X (Lane 7 and 8)

Figure 61
DC-SIGN binds tumor cells that express tumor antigen CEA (CD66e), through
the interaction of DC-SIGN with CD66e.
25   A. DC-SIGNFc recognizes tumor antigens on several breast carcinomas and
adenocarcinomas. Binding can be blocked by anti-DC-SIGN mAbs
B. the tumor cell line SW948 interacts with DC-SIGN expressing cells and
with DCs and the cell adhesion can be blocked by anti-DC-SIGN antibodies
(AZN-D1), indicating a function for DC-SIGN to interact with a cellular
30   structure on tumor cells

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C. DC-SIGN-Fc binds immuneprecipitated CD66e (CEA). Immunoprecipitation with anti-CD66e (anti-CEA) of cell-surface biotinylated SW948 tumor cell-lysate shows that DC-SIGN-Fc binds the tumor antigen CEA (lane 5).

5   Figure 62

DC-SIGN recognizes Lewis X and Lewis Y on CD66e from tumor cells.

A.   CD66e from the tumor cell line SW948 expresses Lewis X and Lewis Y carbohydrate structures s determined using specific antibodies.

B.   Captured CD66e from SW948 lysate is bound by DC-SIGN-Fc

10   C.   DC-SIGN-Fc captures CD66e from a SW948 lysate as detected by an anti-CD66e specific antibody.

D.   Immunoprecipitation (IP) studies revealed that binding of DC-SIGN-Fc to CD66e is abrogated when CD66e is treated with a-1, 3,4 Fucosidase that removes the fucose residue present in Lewis X,Y (Lane 3 and 4).

15

Figure 63.

Schematic picture of different mutants of Neisseria Meningitidis with different end-standing carbohydrate residues on their LPS structures.

20   Figure 64. DC-SIGN binds to the IgtB and rfaF mutants of Neisseria Meningitidis. The different Neisseria Meningitidis mutants are investigated for their interaction using different C-type lectin-Fc molecules (DC-SIGN-Fc, DCIR-Fc, BCDA-2-Fc, MGL-Fc Dectin-1B-Fc) using the Fc ELISA assay.

25   Figure 65 Immature DCs bind strongly to the IgtB Neisseria Meningitidis mutant through DC-SIGN. . Only the fluorescent mutant Neisseria IgtB, that contains an endstanding GlcNAc, binds strongly to DC, as determined by flow-cytometry, and the binding can be completely blocked by anti-DC-SIGN antibodies indicating that DC-SIGN in involved in capture Neisseria of IgtB.

30   Mannose-BSA and mannan partly inhibit the interaction.

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Figure 66. DC-SIGN-Fc strongly binds CHOP8 cells that overexpress GlcNAc, indicating that DC-SIGN also binds GlcNAc residues on self-glycoproteins (CHOP8) and on pathogens such as Neisseria (Figure 33).

5

Examples

Methods.

10 *Antibodies and proteins*. The following monoclonal antibodies (Mab) were used: anti-CD107a (Lamp-1; MabH4A3, BD Pharmingen)., anti-MR (Clone 19, BD Pharmingen), CD11b (bear-1)[25], CD11c (SHCL3)[26], anti-DC-SIGN (AZN-D1, AZN-D2[14], CSRD[10], blocking CD11a (NKI-L15), anti-ICAM-1 (Rek-1), anti-ICAM-2(12A2), and anti-ICAM-3 (AZN-IC3/1, icr-2), activating CD18

15 (KIM185) and the PE/FITC-conjugated antibodies CD25, CD69, CD80, CD86, HLA-DR (BD Pharmingen), and CD83 and CD56 (Beckman Coulter). Biotin-conjugated CD3 and CD16 (BD Pharmingen). CLB-T4 (αCD4) was a kind gift of René van Lier. MEM-157 (αCD16) and MEM-97 (αCD20) were kindly provided by Vaclav Horejsi. ICAM-1 mAb CBR-IC-1/3, ICAM-2 mAb CBR-IC-

20 2/1, and ICAM-3 mAb CBR-IC-3/2 were obtained through the Human Leukocyte Differentiation Antigen Workshop. Several anti-glycan monoclonal antibodies (MAbs) were used: the anti-LDN-DF MAb 114-5B1-A[16], the anti-LDN MAb SMLDN1.1[17], the anti-LDN-F MAb SMLDNF1[10] and the anti-Le[x] MAb CB10[27], the anti-Lex mAbs SMLDN1.1 and SMFG4.1 and6H3 (anti-Lex).

25 The mAb (CLB-gran/10) was used to stain and immunoprecipitate CD16.
        The neoglycoprotein HSA-Le[x], containing approx. 20-25 oligosaccharide chains per HSA molecule, was from Isosep AB, Tullinge, Sweden. The neoglycoprotein BSA-LDN-DF (approx. 12 oligosaccharide chains per molecule BSA), and BSA-LDN-F (approx. 3-4 oligosaccharides per molecule

30 BSA), were synthesized enzymatically as described by van Remoortere et al[16],

50

and Nyame et al[17] , respectively. Le^x-PAA-biotin, containing Le^x multivalently
coupled to biotinylated polyacrylamide was from Syntesome, Munich,
Germany.

5   Mannan purified from *Saccheromyces cerevisiae*   (50 µg/ml) and
recombinant gp120 (0.50 µg/ml) were obtained from Sigma and the
Aidsresource Foundation, respectively. Purified mannose capped
lipoarabinomannan (manLAM) from *Mycobacterium tuberculosis*, and non-
capped LAM (araLAM) from *M. smegmatis* were kindly provided by Dr. J.
Belisle, Colorado State University through NIH, NIAID contract NO1 AI-
10   75320. Soluble egg antige (SEA) was kindly provided by Dr. A. K. Nyame,
Oklahoma University HSC, USA). Purified lipophosphoglycan from
Leishmania mexicana was kindly donated by Dr. M Wiese, Bernard Nocht
Inst. Tropical Medicine Hamburg, Germany. Purified lipopolysaccharide of
Helicobacter pylori was obtained from M. Monteiro, NRC, Ottawa, Canada. A
15   sonicate of bacterial cells of a clinical isolate of *M. tuberculosis* was donated by
A. Kolk, KIT, Royal Trop. Inst, Amsterdam. Clinical isolates of *Helicobacter
pylori, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and
Staphylococcus aureus were obtained from VUMC Hospital, Amsterdam.
Synthetic glycoconjugates were obtained from Syntesome, Munich, Germany,
20   and comprise mono- and oligosaccharides multivalently linked to a
biotinylated polyacrylamide carrier, MW 40.000.

Soluble DC-SIGN-Fc adhesion assay. DC-SIGN-Fc consists of the extracellular
portion of DC-SIGN   (amino acid residues 64-404) fused at the C-terminus to a
25   human IgG1-Fc fragment. DC-SIGN-Fc was produced in Chinese Hamster
Ovary K1 cells by co-transfection of DC-SIGN-Sig-pIgG1 Fc   (20 µg) and
pEE14 (5 µg) vector. DC-SIGN-Fc concentrations in the supernatant were
determined by an anti-IgG1 Fc ELISA. The DC-SIGN-Fc binding assay was
performed as follows. Glycoconjugates and sonicated mycobacteria were coated
30   onto ELISA plates at 5 µg/well; intact bacterial cells were coated at 5×10^7/ml ;

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coating took place for 18 hours at room temperature, followed by blocking
with 1% BSA for 30 min, at 37°C in TSM (20mM Tris-HCl pH 7.4 containing
150mM NaCl, 2 mM CaCl$_2$ and 2mM MgCl$_2$). Soluble DC-SIGN-Fc (approx. 2
µg/ml in TSM buffer) was added and the adhesion was performed for 120 min.

5   at RT. Unbound DC-SIGN-Fc was washed away and binding was determined
by an anti-IgG1 Fc ELISA using a peroxidase conjugate of goat anti-human Fc.
Specificity was determined in the presence of either 20 µg/ml blocking
antibodies, 50 µg/ml mannan or 5mM EGTA.

10   *Cells.* Immature DC were cultured from monocytes in the presence of IL-4 and
GM-CSF (500 and 800 U/ml, respectively; Schering-Plough, Brussels,
Belgium[28]. At day 7 the phenotype of the cultured DC was confirmed by flow
cytometric analysis. The DC expressed high levels of MHC class I and II, αMβ2
(CD11b), αXβ2 (CD11c) and ICAM-1, moderate levels of LFA-1 and CD80, and

15   low levels of CD14. K562 transfectants expressing wild-type DC-SIGN were
generated by transfection of K562 cells with 10 µg pRc/CMV-DC-SIGN plasmid
by electroporation as previously described.

      Stable transfectants of K562 expressing ICAM-3 were obtained by
electroporation of pCDM8-ICAM-3 and pGK-HYG. K562 cells were cultured on

20   RPMI 10% FCS, whereas K562-ICAM-3 cells were cultured on RPMI 10% FCS
: Iscove's 5% FCS 3:1 containing 0.5 mg/ml hygromycine to maintain ICAM-3
expression.

      Naive NK cells were isolated from buffy coats of healthy donors.
Briefly, the PBMC fraction obtained through Ficoll centrifugation was

25   sequentially depleted for CD14$^+$ cells, and CD3$^+$, CD4$^+$, and CD20$^+$ cells using
MACS sorting. CD14$^+$ cells were depleted by CD14 microbeads (Miltenyi
Biotec) on an LS column (Miltenyi Biotec), and CD3$^+$, CD4$^+$, and CD20$^+$ cells
were labeled by the mAbs T3B (αCD3), CLB-T4 (αCD4), MEM97 (αCD20), and
thereafter depleted by goat-α-mouse microbeads (Miltenyi Biotec) on an LD

30   column (Miltenyi Biotec). Thus obtained NK cells were routinely tested for the

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NK cell markers CD16 (75% to 90% expression), CD56 (80 to 95% expression), the non-lineage markers CD3, CD4, CD14 and CD20 (all less than 1% expression), and the early activation marker CD69 (15% expression on non-activated NK cells and 75% expression on 1-day IL-2-activated NK cells).

5   CD56^{dim} and CD56^{bright} NK cell populations were isolated from MACS obtained NK cells by FACS sorting on low and high CD56 expression, respectively. CD56^{dim} and CD56^{bright} NK cell populations were over 95% pure as assessed by flow cytometry staining for CD16 and CD56.

10   *DC activation.* Immature DC (2×10^6 cells/ml) were cultured for 24 hours in the presence of IL-4 (500 U/ml, Schering-Plough, Brussels, Belgium), GM-CSF (800 U/m; Schering-Plough, Brussels, Belgium) and either LPS (10 ng/ml) or LAM glycolipids (15 µg/ml). The effect of LAM on LPS-induced activation was determined by pre-incubating immature DC (300.000 cells) with AZN-D2 (40

15   µg/ml) for 30 minutes, and subsequently with LPS in the presence of LAM (15 µg/ml) for 18 hours. LAM glycolipids were obtained from J. Belisle (Colorado State University and the NIH (contract NO1 AI-75320)) and contained <5 ng/mg endotoxin. Activation was determined by cell-surface expression of MHC class II (HLA-DR) and the co-stimulatory molecules CD80, CD83 and

20   CD86 using PE-conjugated antibodies.

Fluorescent bead adhesion assay. Carboxylate-modified TransFluorSpheres (488/645 nm, 1.0 µm; Molecular Probes, Eugene, OR) were coated with HIV-1 gp120 and ICAM-3 as described[14]. Streptavidin was covalently coupled to the

25   beads as described and streptavidin-coated beads were incubated with biotinylated PAA-linked glycoconjugates (50 pMol; Syntesome, Munich, Germany). The fluorescent bead adhesion assay was performed as described[14]. Ligand-coated fluorescent beads (20 beads/cell) were added to the cells for 45 minutes at 37°C, washed and analyzed by flow cytometry (FACScan, Becton

30   Dickinson, Oxnard, CA), by measuring the percentage of cells that had bound

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fluorescent beads. LAM-coated beads were generated by coating incubating
streptavidin-coated beads were incubated with biotinylated F(ab')2 fragment
goat anti-mouse IgG (6 µg/ml; Jackson Immunoresearch) followed by an
overnight incubation with mouse-anti-LAM antibody (F30.5) at 4°C. The beads

5   were washed and incubated with 250 ng/ml purified glycolipid LAM (obtained
from J. Belisle, Colorado State University and the NIH (contract NO1 AI-
75320)) overnight at 4°C. SEA-coated beads were generated by incubating the
streptavidin-coated beads with biotinylated F(ab')2 fragment of goat anti-
mouse IgG (6 µg/ml; Jackson Immunoresearch), followed by an overnight

10   incubation at 4°C with anti-LDN MAb, or anti-LDN-DF MAb. The beads were
washed and incubated with 1µg/ml SEA overnight at 4°C. Essentially, $50x10^3$
cells were pre-incubated in adhesion buffer (20 mM Tris-HCl pH 8.0, 150 mM
NaCl, 1mM CaCl2, 2 mM MgCl2, 0.5% BSA) with or without blocking MAbs
(20 µg/ml) or mannan (50 µg/ml) for 10 minutes at room temperature. Ligand-

15   coated fluorescent beads (20 beads/cell) were added to the cells and the
suspension was incubated for 45 minutes at 37°C. Cells were washed and
adhesion was determined using flow cytometry (FACScan, Becton Dickinson,
Oxnard, CA), by measuring the percentage of cells that had bound fluorescent
beads. HIV-1 gp120 fluorescent beads were prepared as described previously[19].

20

DC-SIGN-Fc adhesion. Cells were incubated with DC-SIGN-Fc for 30 minutes
at 37°C under saturating conditions (concentration: 10 µg/ml), and
subsequently with FITC-conjugated goat-α-human secondary antibodies to

25   monitor adhesion of DC-SIGN-Fc. Before incubation with cells, DC-SIGN-Fc
was preincubated for 10 min at RT either with medium, aDC-SIGN (AZN-D1,
50 µg/ml), mannan (50 µg/ml), or EGTA (10 mM) to determine specificity of
DC-SIGN-Fc adhesion. DC-SIGN-Fc adhesion was determined by flow
cytometry (FACS Calibur, Beckman Coulter).

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Mycobacteria. Both the *M. bovis* BCG (Pasteur) and the *M.tuberculosis* H37Ra strains were gifts from A. Kolk (Royal Tropical Institute, Amsterdam). *M. bovis* BCG was cultured *in vitro* using Middelbrook 7H9 broth supplemented with 0.05% Tween 80 and albumin-dextrose-catalase. The glycolipids ManLAM

5   and AraLAM were obtained from J. Belisle, Colorado State University and the NIH (contract NO1 AI-75320). DC were infected with mycobacteria by co-culturing them at an appropriate multiplicity of infection (MOI) as indicated in the figure legends.

10   *Fluorescent mycobacterial binding assay.* Capture and internalization of mycobacteria by cells was evaluated using fluorescein isothiocyanate (FITC)-conjugated *M. bovis* BCG. Bacteria (10⁹/ml) were labeled by incubation of 0.5 mg FITC per ml in phosphate buffered saline (pH 7.4) at room temperature for 1 hour. The FITC-pulsed bacteria were washed three times to remove unbound

15   FITC. Capture was determined by measuring the percentage of cells that bound FITC-conjugated bacteria using flow cytometry (FACScalibur, Becton Dickinson Immunocytometry, San Jose, CA). Phagocytosis was determined using a fluorescence-quenching technique as reported previously[29]. In brief, quenching of non-internalized membrane-bound FITC-conjugated *M. bovis*

20   BCG was achieved by treating the cells with 0.05% trypan blue for 5 minutes. *H. pylori* binding was assessed by labeling the bacteria with FITC and binding to DC was investigated similarly to the bead assay.

Cytokine production. For the detection of cytokines, culture supernatants were

25   harvested at day 1 and frozen at -80°C until analysis. The supernatants were analyzed for the presence of IL-10 and IL-12p40 by ELISA (Biosource International, CA).

Defucosylation of SEA. SEA were defucosylated by incubation of the antigens

30   at 100°C for 1 hour in 0.1M TFA. After neutralization, the defucosylated

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antigen was coated to ELISA plates. Enzymatic defucosylation was performed by incubating the SEA in 50 mM sodiumphosphate pH=5.0 with α1,3/4-fucosidase (0.4 mU/µg SEA) (Calbiochem) overnight at 37°C. The degree of defucosylation of the antigens was assessed by their ability to bind MAbs

5 specifically recognizing the fucosylated glycan epitopes LDN-DF and Le^x, whereas the integrety of other, non-fucosylated, glycan epitopes was assessed by measuring the reactivity with anti-LDN MAb.

SDS-PAGE, Western blotting and silver staining. SEA were separated by SDS-

10 PAGE under reducing conditions on a 12,5% polyacrylamide gel, using the Mini-Protean II system (BioRad), and proteins visualized by silver-staining. For Western blotting proteins were transferred onto a nitrocellulose membrane (Schleicher and Schuell). The membrane was blocked in a solution of 5% BSA in TSM for 2 h followed by incubation in 2 µg/ml DC-SIGN-Fc in TSM buffer

15 containing 1% BSA for 1 h. After washing, the membrane was subsequently incubated for 1 h in peroxidase conjugated goat anti-human IgG1 and reactive bands were visualized by detection with CN/DAB substrate (Pierce).

Cytotoxicity. The standard 4-hour ^{51}Cr release assay was used to assess NK

20 cell-mediated cytotoxicity. Briefly, $1*10^6$ target cells were labeled by 100 µCi ^{51}Cr for 1 hour at 37°C, extensively washed to remove free ^{51}Cr, resuspended at 2500 cells/well (iDC and mDC) or 1000 cells/well (K562 and K562-DC-SIGN), and incubated with NK cells for 4 hours at 37°C at the indicated ratios and under the indicated conditions. After 4 hours scintillation liquid

25 (PerkinElmer) was added to supernatants, and ^{51}Cr release was determined on a micro-b counter (PerkinElmer).

NK cell-mediated DC maturation. Resting and activated NK cells were obtained by overnight incubation on medium and IL-2 (1000 U/ml),

30 respectively. Thereafter, resting and activated NK cells were incubated

overnight with immature DC in a 96 wells U-bottom plate (Costar) on RPMI 10% FCS. NK cells were preincubated for 10 min. at RT with medium or blocking αLFA-1 mAbs (NKI-L15, 50 μg/ml), whereas DC were preincubated with medium or blocking αDC-SIGN mAbs (AZN-D2, 50 μg/ml). As a positive

5　　control DC were incubated overnight in the presence of LPS (2 μg/ml). Maturation of DC was assessed by flow cytometry (FACS Scan, Beckman Coulter) for the maturation markers CD80, CD83, CD86, and HLA-DR by PE-conjugated or FITC-conjugated mAbs.

10　　*DC-induced NK cell activation.* Resting NK cells were incubated for 2 days with immature or mature DC (obtained by LPS maturation) in a 96 wells U-bottom plate (Costar) on RPMI 10% FCS. LFA-1-dependent DC-induced NK cell activation was determined by a 10 min. preincubation of NK cells with αLFA-1 mAbs (NKI-L15, 50 μg/ml), whereas dependency on DC-SIGN was

15　　assessed by a 10 min. preincubation of DC with anti-DC-SIGN mAbs (AZN-D1 and AZN-D2, 50 μg/ml). As a positive control NK cells were incubated for 2 days with IL-2 (1000 U/ml). NK cell activation was assessed by flow cytometry (FACS Calibur, Beckman Coulter) for the early activation marker CD69 by FITC-conjugated mAbs.

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Immunoprecipitation. NK cells were surface iodinated with 1 mCi ^{125}I, or biotinylated and subsequently lysed in lysisbuffer (1% Triton-X -100, 10 mM TEA, 150 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 1 mM PMSF, 20 μg/ml trypsin inhibitor, 20 μg/ml leupeptin, and 20 μg/ml aprotinin). DC-SIGN ligands,

25　　ICAM-2, ICAM-3, and LFA-1 were immunoprecipitated from prot A-precleared NK cell lysate by prot A beads covalently linked to DC-SIGN-Fc, αICAM-2 (12A2), αICAM-3 (AZN-IC-3/1), and αLFA-1 (NKI-L15). Immunoprecipitates were reduced in sample buffer (containing 4% SDS and 5% b-mercapto-ethanol), heated for 5 min at 95°C, and run on a 5-15% gradient

30　　polyacrylamide gel (SDS-PAGE).

Alternatively, specific anti-bodies against CD66acd or ICAM-3 or LFA-1 were coated in an ELISA plate, subsequently lysates were added and incubated for 1 hr at 4C to retract the specific proteins. DC-SIGNFc was incubated for binding activity to the specific proteins and stained with Goat anti human Fc
5   PO and developed.

Experimental setup.

10   Dendritic cells (DC) are instrumental in handling pathogens for processing and presentation to T cells, thus eliciting an appropriate immune response. C-type lectins expressed by DC function as pathogen-recognition receptors; yet their specificity for carbohydrate structures on pathogens is not fully understood. Here we analyzed the carbohydrate specificity of DC-
15   SIGN/CD209, the recently documented HIV-1 receptor on DC. Our studies show that DC-SIGN binds with high affinity to both synthetic mannose- and fucose- containing glycoconjugates. These carbohydrate structures are abundantly expressed by pathogens as demonstrated by the affinity of DC-SIGN for natural surface glycans of the human pathogens *Mycobacterium*
20   *tuberculosis, Helicobacter pylori, Leishmania mexicana* and *Schistosoma mansoni*. Strikingly, these pathogens target DC-SIGN to infect DC but also to modulate the immune responses mediated by DC.

This analysis expands our knowledge on the carbohydrate and pathogen-specificity of DC-SIGN, and identifies this lectin to be central in
25   pathogen-DC interactions.

Example 1: Novel Carbohydrate specificity for DC-SIGN

For *in vitro* binding studies we generated a chimeric protein of DC-
30   SIGN with a human IgG1 Fc tag, which we used to screen in an ELISA format

for reactivity with a panel of synthetic glycoconjugates containing mannose or fucose residues and their derivatives in multimeric form.

As reported earlier, DC-SIGN-Fc binds to purified yeast-derived mannan and the high mannose containing HIV-1 gp120, but also to less

5   complex mannose-containing glycoconjugates i.e. mannose and α1->3, α1->6mannotriose (Table I, Figure 1). Strikingly, DC-SIGN binds to Lewis blood group antigens (Le^x, Le^y, Le^a, Le^b), glycan comprising at least one terminal fucose α1,3 or α1,4-linked to N-acetylglucosamine (Le^x, Le^y Le^a, Le^b, LDNF), or □-1,2 to Galactose (Le^y, Le^b) (Table I, Figure1). Sialylation of Le^x

10   (yielding sialyl-Le^x, a L-, E- and P-selectin ligand) completely abrogates the recognition by DC-SIGN, indicating that DC-SIGN has a carbohydrate specificity that is distinct from that of the selectins that mediate leukocyte rolling. Sulfation reduced the binding affinity of DC-SIGN for Le^x, as well as Le^a (Figure 1c). To compare in more detail the affinity of DC-SIGN binding to

15   Le^x and □1>3, □1>6-mannotriose, titration studies were performed with the different DC-SIGN binding glycoconjugates (Figure 1c). Strikingly, DC-SIGN binds with much higher affinity to the fucose-containing carbohydrate Le^x than to mannotriose. The binding activity of DC-SIGN-Fc to these glycan structures was specific, since anti-DC-SIGN antibodies blocked the interaction (Figure

20   1c).

To determine whether DC-SIGN-Fc exhibits a similar carbohydrate recognition profile as cell-surface expressed DC-SIGN, both DC-SIGN transfectants and monocyte-derived DC were studied for carbohydrate binding activity using a fluorescent beads adhesion assay with different

25   glycoconjugates (α1->3, α1->6mannotriose, Le^x and sulfo-Le^a) (Figure 2). Indeed, DC-SIGN expressed by K562 transfectants bound similarly to the glycoconjugates as DC-SIGN-Fc and the binding was completely inhibited by anti-DC-SIGN antibodies (Figure 2). Even though DC express many other C-type lectins on their cell surface, our data demonstrate that the

30   glycoconjugates containing Le^x and α1->3, α1->6mannotriose are preferentially

59

bound by DC-SIGN. The interaction is specific since anti-DC-SIGN antibodies
almost completely inhibited the binding activity. This illustrates that DC-
SIGN is the major receptor on DC for these carbohydrate structures. Binding
of sulfo-Le[a] to DC could only be partially blocked by anti-DC-SIGN antibodies

5   indicating that other C-type lectins on DC compete with DC-SIGN for binding
of sulfo-Le[a] . Our data show that DC-SIGN recognizes a wider range of glycan
structures, including Lewis blood group antigens, than hitherto realized. Thus,
DC-SIGN may be an important receptor for recognition of novel biologically
relevant targets expressed by the host, or alternatively by human pathogens.

10

Example 2. Novel carbohydrate specificity (Le[x] and high mannose) identifies
novel pathogens (*Helicobacter pylori, Schistosoma mansoni, Mycobacterium
tuberculosis, Leishmania mexicana*) that interact with DC-SIGN

15   We subsequently investigated the binding of DC-SIGN to human
pathogens that express mannose- or fucose-containing glycans. The gram-
negative *Helicobacter pylori* bacterium, which induces peptic ulcers and gastric
carcinoma[20], and the worm parasite (the causal agent of schistosomiasis) both
express Le[x] [21]. In *H. pylori*, Le[x] is present on surface-located

20   lipopolysaccharide, while in *S. mansoni* Le[x] is expressed by all stages of the
parasite, including soluble egg antigen (SEA)[21]. Binding of DC-SIGN-Fc to
Le[x]-positive *H. pylori* lysate and to extract of *S. mansoni* was strong and was
completely inhibited by anti-DC-SIGN antibodies (Figure 3); When analysisng
two different LPS forms from Klebsiella one containing mannose cap and one

25   without (Len1 and Len1II respectively) (Figure 3B). DC-SIGN expressed by
DC also bound to purified LPS of *H. pylori* (Figure 4). Binding of DC to both *H.
pylori* cells and purified LPS could be completely blocked with anti-DC-SIGN
Mab (Figure 4). Also glycans present in *Mycobacterium tuberculosis*, the
causative agent of tuberculosis, are bound by DC-SIGN (Figure 3). The

30   mannose-capped surface glycan, lipoarabinomannan of *Mycobacterium*

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tuberculosis probably contains the recognition site for DC-SIGN. This is further supported by the fact that DC-SIGN also bound to the mannose-capped surface lipophosphoglycan (LPG) expressed by an unicellular parasite that causes leishmaniasis (Figure 3). Binding of DC-SIGN to *Leishmania* was

5   reported very recently[30] but we demonstrate here that LPG is the structure on *Leishmania* that is recognized by DC-SIGN (Figure 3). No binding of DC-SIGN to three clinically relevant Gram-negative bacterial human pathogens (*Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa*) was observed nor to Gram-positive *Staphylococcus aureus*. However other clinical

10   isolates of Klebsiella that contain a mannose capped LPS bind DC-SIGN. These findings indicate that binding of DC-SIGN to pathogens is selective, and that the carbohydrate specificity of DC-SIGN governs a broader pathogen recognition than only viruses such as HIV-1 and Ebola virus[12,22].

15   Example 3 DC-SIGN interacts with *M. tuberculosis* through ManLAM glycolipids

Whole mycobacteria such as the *M. tuberculosis* H37Ra strain and *M. bovis bacillus* Calmette-Guérin (BCG) were coated and the interaction of

20   DC-SIGN with these pathogens was analyzed using the DC-SIGN-Fc binding assay. *M. bovis* BCG is a tuberculosis strain that is almost nonpathogenic yet retains the immunological properties of tuberculosis. DC-SIGN-Fc interacted specifically with both *M. tuberculosis* H37Ra and *M. bovis* BCG, since the interaction was inhibited with blocking DC-SIGN-specific antibodies (Figure

25   6a). *M. smegmatis*, an avirulent strain that does not contain a Mannose cap, did not bind DC-SIGN (data not shown).
Moreover, an irrelevant Fc chimera, ICAM-3-Fc, did not interact with the mycobacteria (Figure 6a). The interaction is mediated by the C-type lectin domain of DC-SIGN, since binding to both *M. tuberculosis* and *M. bovis* BCG

was inhibited by EGTA, mannan and the DC-SIGN-specific antibody AZN-D1 that recognizes the lectin domain[23] (Figure 6a).

We next investigated the binding of DC-SIGN to purified mycobacterial lipoarabinomannan (LAM), since DC-SIGN has a high affinity

5   for mannose-containing carbohydrates and LAM is the major mannose-containing component of the mycobacterial cell-wall[31]. LAM comprises a mannose-rich polysaccharide-core, containing highly branched arabinofuranosyl side chains, and a GPI anchor (Figure 6b). LAM isolated from *M. tuberculosis* contains mannose-residues consisting exclusively of

10   mono-, di- and trimers of □-D-mannoses directly linked to the arabinofuranosyl-termini and is called ManLAM, whereas LAM isolated from the fast growing *M. smegmatis* is not mannose-capped and is called AraLAM[31] (Figure 6b). Strikingly, purified ManLAM was efficiently bound by DC-SIGN, in contrast to AraLAM (Figure 6c), demonstrating that DC-SIGN specifically

15   interacts with the mono-, di- and trimers of α-D-mannoses of ManLAM. Even at high concentrations, DC-SIGN did not bind AraLAM, demonstrating a high specificity for ManLAM and its mannose-cap (Figure 6d). The interaction of DC-SIGN to ManLAM is specific, since the binding was inhibited by antibodies against DC-SIGN, whereas an irrelevant Fc chimera did not interact with

20   ManLAM (Figure 6c). DC-SIGN interacts similarly with both ManLAM and whole *M. bovis* BCG indicating that DC-SIGN binds mycobacteria through ManLAM (Figure 6a and c).

Example 4 Both mycobacteria and ManLAM interact with the primary binding

25   site of DC-SIGN

We used K562 transfectants stably expressing DC-SIGN to investigate the binding of cell-surface-expressed DC-SIGN to both *M. bovis* BCG and the mycobacterial component ManLAM. These cells do not express

30   the previously reported mycobacterial receptors Mannose Receptor (MR),

62

CD11b and CD11c (Figure 7a). K562 transfectants express high levels of DC-SIGN (Figure 7a) and bind strongly to both *M. bovis* BCG and ManLAM, in contrast to mock transfected K562 cells (Figure 7b). The interaction is blocked by DC-SIGN-specific antibodies (Figure 7b). The interaction of DC-SIGN with both *M. bovis* BCG and ManLAM is similar to that of the other DC-SIGN ligands ICAM-3 and HIV-1 (Figure 7b). Thus, cellular DC-SIGN specifically binds to both *M. bovis* BCG and ManLAM, as was observed with recombinant DC-SIGN-Fc (Figure 6c).

The C-type lectin domain of DC-SIGN contains two calcium ions, and the amino acid residues that are in close contact with Ca^{2+} at site 2 (Glu^{347}, Asn^{349}, Glu^{354} and Asn^{365}) form the core of the ligand binding site[23]. Changing in DC-SIGN either Glu^{347} into Gln (E347Q), or Asn^{349} and Asn^{365} into Asp, resulted in complete loss of binding to whole mycobacteria and ManLAM (Figure 7b and data not shown), similarly as was shown previously for both ICAM-3 and HIV-1 gp120 (Figure 7b). The Ca^{2+} at site 1, the so-called auxiliary site, coordinates the correct positioning of the primary binding site, and loss of this Ca^{2+} by mutating Asp^{320}, Glu^{324} (E324A), Asn^{350} or Asp^{355} into Ala residues resulted in complete loss of both *M. bovis* BCG and ManLAM binding (Figure 7b and results not shown).

Recently, we demonstrated that the binding site of DC-SIGN for its cellular ligand ICAM-3 is distinct from that of HIV-1 gp120, since a specific mutation in DC-SIGN (V351G) abrogated ICAM-3, but not HIV-1 gp120 binding (Figure 7c). Strikingly, the DC-SIGN V351G mutant also interacts with *M. bovis* BCG as well as ManLAM (Figure 7c), demonstrating that both HIV-1 and mycobacteria bind similarly to DC-SIGN at a distinct site from the cellular ligand ICAM-3. The similar binding of both *M. bovis* BCG and ManLAM to DC-SIGN mutants further supports our findings that DC-SIGN specifically interacts with ManLAM on whole mycobacteria. Binding of fluorescent beads coated with distinct neoglycoproteins of ManLAM that consist of Arabinose, Arabinose-□1,5 mannose, Arabinose-□□1,5Man-□□1,2

63

Man, Ara-☐☐1,5Man-☐☐1,2Man-☐☐1,2Man or Ara6 demonstrate that DC-SIGN particularly recognizes in ManLam a di-mannose (Man ☐1,2 component) or mannosetriose (Man ☐1,2 component) (Figure 7D).

5 Exampe 5 DC-SIGN is a major receptor for mycobacteria on DC

    Immature DC express, besides high levels of DC-SIGN, high levels of the receptors MR, CD11b and CD11c (Figure 8a), which have previously been reported to mediate binding of mycobacteria by macrophages. We used
10 blocking antibodies against these receptors to evaluate their contributions to ManLAM binding by DC. Immature DC bind strongly to ManLAM, but not to AraLAM, and the interaction was inhibited by the DC-SIGN-specific antibody, but strikingly not by any of the antibodies against MR, CD11b or CD11c (Figure 8b). Both EGTA and the C-type lectin-specific inhibitor mannan block
15 ManLAM binding by DC to a similar extent as the DC-SIGN-specific antibodies, demonstrating that DC-SIGN is the primary ManLAM-binding C-type lectin on immature DC that binds ManLAM (Figure 8b).

    The major contribution of DC-SIGN to the interaction of immature DC to ManLAM prompted us to investigate whether DC-SIGN could be
20 important in the interaction of immature DC to whole mycobacteria. Immature DC strongly interacted with *M. bovis* BCG (Figure 8c). Strikingly, DC-SIGN is a major receptor for *M. bovis* BCG, since the antibodies against DC-SIGN strongly inhibited the infection of immature DC with *M. bovis* BCG (Figure 8c). Antibodies against MR, CD11b and CD11c did not inhibit the infection,
25 whereas the C-type lectin inhibitor mannan blocked the infection to a similar level as the DC-SIGN antibodies (Figure 8c). Moreover, the MR-ligand mannose-BSA did not inhibit the interaction of DC with *M. bovis* BCG (Figure 8c) demonstrating that the C-type lectin domains of MR are not involved in *M. bovis* BCG infection of DC. Both the anti-MR antibody and mannose-BSA are
30 inhibitors of MR-function since they block binding of another MR-ligand

dextran to DC (results not shown). These results demonstrate that DC-SIGN is the primary C-type lectin on DC that functions as a receptor for *M. bovis* BCG. Other non-lectin receptors may participate in the interaction since the infection was not completely inhibited by antibodies against DC-SIGN (Figure

5 8c).

Example 6 DC-SIGN facilitates capture and internalization of *M. bovis* BCG by immature DC through binding of the mycobacterial cell-wall component ManLAM.

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Next we investigated whether DC-SIGN mediates internalization of *M. bovis* BCG using trypan blue to quench surface FITC-conjugated mycobacteria. Mock transfected K562 cells did not phagocytose *M. bovis* BCG, whereas 50% of the K562 transfectant, expressing DC-SIGN, had internalized

15 *M. bovis* BCG within 45 minutes (Figure 8d). Both mannan and antibodies against DC-SIGN blocked the internalization of *M. bovis* BCG (Figure 8d).

Immature DC are highly phagocytosing cells and indeed within 45 minutes more than 90% of the dendritic cells that bound *M. bovis* BCG, had internalized the mycobacteria (Figure 8e). Similar as was observed for the

20 binding of *M. bovis* BCG by DC (Figure 8c), phagocytosis of *M. bovis* BCG is partly blocked by antibodies against DC-SIGN whereas both anti-MR antibodies and the MR-ligand mannose-BSA did not inhibit the observed phagocytosis (Figure 8e). These results demonstrate that DC-SIGN facilitates capture and internalization of *M. bovis* BCG by immature DC through binding

25 of the mycobacterial cell-wall component ManLAM.

Example 7. Mycobacteria and ManLAM are internalized by DC-SIGN and targeted to lysosomes

Recently, we have demonstrated that DC-SIGN can function as an antigen receptor that internalizes antigens and targets them to lysosomal compartments for presentation on MHC Class II[10]. Therefore, the fate of the captured *M. bovis* BCG by immature DC was followed by immuno-fluorescence

5   analyses. Immature DC were incubated with FITC-conjugated *M. bovis* BCG for 2 hours and both DC-SIGN and Lamp-1 were stained (Figure 9a). The observed co-localization of DC-SIGN with FITC-conjugated *M. bovis* BCG further supports a role for DC-SIGN in the capture and internalization of mycobacteria (Figure 9a). Phagocytosed mycobacteria are targeted to the

10   lysosomes since the internalized FITC-conjugated mycobacteria co-localize with Lamp-1 staining (Figure 9a). Similarly, ManLAM was also captured and internalized by DC-SIGN on immature DC since ManLAM staining co-localized with DC-SIGN (Figure 9a) whereas AraLAM was not internalized by DC (results not shown). Internalized ManLAM co-localized with the lysosomal

15   marker LAMP-1/CD107a in immature DC (Figure 9a) indicating that internalized ManLAM is targeted to lysosomes. Thus, both whole mycobacteria and the cell-wall component ManLAM are similarly internalized by immature DC through DC-SIGN, supporting the results that DC-SIGN interacts with ManLAM on mycobacteria.

20   Recently, we demonstrated that binding of soluble ligands or antibodies to DC-SIGN triggers internalization of the DC-SIGN-ligand complex to the late endosomes/lysosomes, and results in down-regulation of DC-SIGN from the surface[10]. Therefore, we investigated whether DC-SIGN is internalized after ManLAM binding by measuring the cell-surface expression

25   of DC-SIGN after ManLAM binding using a specific antibody against DC-SIGN. Indeed, binding of ManLAM, but not AraLAM, to DC-SIGN results in down-regulation of DC-SIGN (Figure 9b), demonstrating that DC-SIGN on DC binds ManLAM and mediates the internalization of ManLAM to CD107a^{+} lysosomes. Moreover, other mycobacteria-receptors such as MR, CD11b and

30   CD11c were not downregulated (Figure 9b). Thus, ManLAM binding to DC-

SIGN triggers internalization of the DC-SIGN/ManLAM complex, and might enable antigen processing of ManLAM by DC.

Example 8. ManLAM changes the cytokine production by DC through DC-
5   SIGN

ManLAM is present not only a mycobacterial cell-wall component but is also secreted from phagosomes following macrophage ingestion of M. tuberculosis[31]. Potentially, mycobacteria within infected macrophages can
10   influence bystander immune cells and modulate the immune response through secretion of ManLAM. The cytokine IL-10 is a potent immunosuppressive factor induced in macrophages by some intracellular bacteria to dampen down host immune responses and promote their survival[32]. We investigated the influence of ManLAM binding to DC-SIGN in IL-10 production by DC.
15   ManLAM alone did not induce IL-10 production by immature DC (Figure 10a). Strikingly, ManLAM, but not AraLAM, strongly induced IL-10 production by DC, when they received simultaneously an activation signal, such as LPS (Figure 10a). This IL-10 induction was completely inhibited by DC-SIGN-specific antibodies to the level of LPS-activated DC alone (Figure 10a). The
20   findings that only ManLAM could induce IL-10 production, which could be blocked by DC-SIGN-specific antibodies indicates that the IL-10 induction is specific for the ManLAM/DC-SIGN interaction. Antibodies against DC-SIGN alone did not induce IL-10 production by LPS-activated DC (Figure 10a), demonstrating that ligation of DC-SIGN alone is not sufficient for IL-10
25   induction. Thus, the binding of ManLAM to DC-SIGN triggers intracellular signals that induce IL-10 production by DC, indicating that mycobacteria target DC-SIGN to suppress the immune response and promote their survival in the host. Both immature and LPS-activated DC, alone or in combination with ManLAM, produced very low amounts of IL-12p70 (<5 pg/ml). Infection of
30   immature DC with M. bovis BCG induced a strong production of IL-10 that

was not inhibited by antibodies against DC-SIGN (Figure 10b). No differences were observed in the presence of ManLAM (Figure 10b). These results suggest that mycobacteria induce IL-10 production by direct infection as well as by secreting ManLAM.

Example 9. ManLAM inhibits TLR4-mediated DC activation through DC-SIGN

Immature DC are highly efficient in antigen capture and processing, whereas mature DC are specialized in the naïve T cell activation necessary for cellular immune responses. Immature DC mature in response to specific 'danger' signals such as bacterial components (LPS) or inflammatory cytokines (TNFa, PGE2). We investigated the effect of ManLAM on the maturation of DC. Neither ManLAM nor AraLAM induced DC maturation, since both ManLAM and AraLAM, in contrast to LPS that triggers TLR4, did not up-regulate expression of the activation markers CD80, CD83, CD86 or HLA-DR (Figure 11a).

Acute mycobacterial infections represent sites of inflammation that attract and induce DC maturation through the presence of maturation components. Therefore, we investigated the effect of ManLAM and AraLAM in combination with the stimulatory bacterial LPS. Toll-like receptor-4 (TLR4) interaction with LPS generates intracellular signaling, most notably via the transcription factor NFkB, that results in DC activation/maturation. Indeed, DC efficiently mature in the presence of LPS alone (Figure 11b). Strikingly, this LPS-induced activation is inhibited in the presence of ManLAM, since the expression levels of the activation markers CD80, CD83 and CD86 were considerably lower than those of LPS-activated DC (Figure 11b). The observed inhibition of DC activation/maturation is specific for ManLAM, since AraLAM did not inhibit DC activation (Figure 11b). This is further supported by the

ability of antibodies against DC-SIGN, that inhibit ManLAM binding, to

completely restore LPS-induced maturation in the presence of ManLAM

(Figure 11b). These results indicate that ManLAM binding to DC-SIGN

generates intracellular signals that interfere with the TLR4-mediated

5   activation of DC. Moreover, this process is specific for the ManLAM-DC-SIGN

interaction and DC-SIGN ligation alone is not sufficient since antibodies

against DC-SIGN did not block LPS-induced DC activation/maturation (Figure

11b).

10   Example 10. *M. bovis* BCG-induced DC maturation is inhibited by ManLAM.

Both *M. tuberculosis* and *M. bovis* BCG are able to induce DC

maturation through their cell-wall components via TLR2- and TLR4-mediated

signaling[33-36]. Indeed, *M. bovis* BCG infection of immature DC results in DC

15   maturation, as demonstrated by the increased expression of MHC class II and

the co-stimulatory molecules CD80, CD83 and CD86 after *M. bovis* BCG

infection (Figure 12a). Next, we investigated whether ManLAM binding to DC-

SIGN prevented *M. bovis* BCG-induced DC maturation, since immature DC

attracted to sites of mycobacterial infection will encounter both secreted

20   ManLAM and intact mycobacteria. Strikingly, the *M. bovis* BCG-induced DC

maturation is inhibited strongly by ManLAM (Figure 12b). The expression of

MHC class II, CD80, CD83 and CD86 on *M. bovis* BCG-infected DC in the

presence of ManLAM was considerably lower than on *M. bovis* BCG-infected

DC (Figure 12b). Moreover, the maturation was mostly restored when DC were

25   pre-incubated with the blocking DC-SIGN-specific antibody (Figure 12b),

demonstrating that the ManLAM interaction with DC-SIGN prevents DC

maturation by *M. bovis* BCG. Moreover, AraLAM did not block the DC

maturation by *M. bovis* BCG (Figure 11c), since the co-stimulatory molecules,

CD80, CD83 and CD86, are expressed at similar levels on both infected DC

30   and AraLAM-treated infected DC. This indicates that the DC-SIGN-ManLAM

interaction blocks the maturation of DC induced by LPS as well as *M. bovis*
BCG.

Example 11. *S. mansoni* soluble egg antigens (SEA) bind to human immature
5   dendritic cells (DC) through interaction with DC-SIGN.

Because DC are central in directing Th1-Th2 responses, we searched
for a cell-surface receptor expressed on DC that interacts with *S. mansoni*
SEA. To detect binding of SEA to human immature DC, a fluorescent bead
10   adhesion assay was developed. Fluorescent beads were pre-coated with
monoclonal antibodies (MAbs) to SEA glycan antigens and then used to
capture SEA. The conjugated beads were allowed to interact with DC. SEA is
a mixture of glycoproteins, containing many immunogenic glycan antigens (3,
36). Major glycan antigens present in SEA, and the recognition of these
15   antigens by anti-glycan MAbs, are depicted in Figure 13. To capture SEA on
the fluorescent beads, we used MAbs against GalNAcß1,4GlcNAc (LDN) and
GalNAcß1,4(Fuc□1,2Fuc□1,3)GlcNAc (LDN-DF) (Figure 13), both epitopes
that occur on many glycoconjugates within SEA and which are absent on DC.
Strong binding of SEA to DC was observed with SEA coated fluorescent beads
20   that were coupled via anti-LDN MAb as well as via anti-LDN-DF MAb (Figure
14b). Binding of SEA to DC was comparable in strength to the binding of HIV-
1 gp120 to DC. Because the C-type lectins DC-SIGN and the MR, that are
expressed by immature DC (Figure 14a), are potential receptors for the
recognition of glycan antigens, we investigated whether antibodies directed
25   against the C-type lectin CRD of these molecules, or mannan hapten could
inhibit binding of SEA to the DC. The binding of SEA to DC was strongly
blocked both by the anti-DC-SIGN MAb AZN-D1, that binds to the CRD of DC-
SIGN and by mannan, but not by the anti-MR MAb clone 19 (Figure 14b).
These data indicate that DC-SIGN is not only a pathogen receptor on DC for
30   HIV-1, but also for Schistosome SEA.

70

Example 12. A sub-fraction of SEA contains high affinity ligands for DC-SIGN.

To further analyze the binding properties of DC-SIGN to *S. mansoni*
5   SEA, we investigated binding of soluble chimeric DC-SIGN-Fc to SEA. In an
ELISA based assay DC-SIGN-Fc showed efficient binding to wells coated with
SEA (Figure 15a). Binding of DC-SIGN-Fc to SEA is mediated by the CRD of
DC-SIGN, since the interaction was completely inhibited by the anti-DC-SIGN
antibody AZN-D1, or EGTA that removes Ca^{2+} ions that are essential for
10   carbohydrate binding. Binding of DC-SIGN-Fc to SEA is likely to be high
affinity, since binding was observed at very low coating concentrations of SEA
(Figure 16). Because SEA contains many different glycoproteins, we
investigated whether a subset of glycoproteins within SEA interact with DC-
SIGN. SEA glycoproteins were separated by SDS-PAGE and analyzed by
15   Western blotting with DC-SIGN-Fc and anti-glycan antibodies reactive with
Le[x] and LDN-DF, respectively. Among the proteins present in SEA a major
protein of approximately 70-80 kD, and two minor high-molecular proteins
showed interaction with soluble DC-SIGN-Fc (Figure 17). Remarkably, some
glycoproteins of similar apparent molecular weight as those reacting with DC-
20   SIGN-Fc bound to MAbs specific for Le[x] and LDN-DF glycans.

Example 13. DC-SIGN binds to α3-fucosylated glycans.

Many glycoproteins of SEA are heavily fucosylated (3, 36). Because
25   it has been reported that DC-SIGN may exhibit binding to both mannose and
fucose, and Western blotting of SEA suggested that Le[x] and/or LDN-DF
glycans are present on SEA proteins with similar apparent MW as the SEA
proteins binding to DC-SIGN, we explored whether defucosylation of SEA
affects its recognition by DC-SIGN-Fc. Treatment of SEA with the
30   Xanthomonas ☐1,3/1,4-fucosidase resulted in 50% decreased reactivity of anti-

71

Le^x MAb to SEA, whereas no loss in reactivity was observed, as expected, with anti-LDN-DF Mab. The LDN-DF epitope contains terminal α2-linked fucose. The results thus indicate that the that the glycosidase treatment specifically removed part of the α3-fucose moieties present on SEA. The loss in a3/4-fucose

5   residues in SEA upon treatment with the Xanthomonas α1,3/α1,4-fucosidase also resulted in a 25% loss of binding of DC-SIGN-Fc (results not shown). These data suggest that α1,3/α1,4-linked fucose residues may be important for binding of soluble DC-SIGN-Fc to SEA.

Next we investigated whether one or more of the fucosylated SEA

10   antigens Le^x, LDNF or LDN-DF (see Figure 13) may function as ligand(s) for DC-SIGN on SEA. We analyzed the potential of DC-SIGN-Fc to bind to neoglycoproteins containing these glycan antigens in ELISA. The results show that DC-SIGN-Fc strongly interacts with the neoglycoprotein HSA-Le^x and to a lesser extent with BSA-LDNF, which both carry a terminal α3-fucose. By

15   contrast, DC-SIGN-Fc does not bind BSA-LDN-DF in which the α3-fucose is capped with an α2-fucose (Figure 18). The binding is fucose-dependent, since no binding was observed to neoglycoproteins carrying Galβ1,4GlcNAc (LN) or GalNAcβ1,4GlcNAc (LDN). The binding to Le^x and LDNF by DC-SIGN is mediated through its CRD, since binding was inhibited by anti-DC-SIGN and

20   EDTA. In conclusion, these results indicate that DC-SIGN strongly recognizes the α3-fucosylated trisaccharide Le^x and most likely interacts with α3-fucosylated glycans on SEA such as Le^x and/or LDNF.

Example 14. The amino acid residue Val^{351} within DC-SIGN is crucial for

25   binding of DC-SIGN to both SEA and Le^x

The C-type lectin domain of DC-SIGN binds two Ca^{2+} ions and those amino acid residues in close contact with Ca^{2+} at site 2 (Glu^{347}, Asn^{349}, Glu^{354} and Asn^{365}) or with Ca^{2+} at site 1 (Asp^{320}, Glu^{324}, Asn^{350} and Asp^{355}) are

30   essential for ligand binding[37]. Because the results above indicated that the α3-

fucosylated trisaccharide Le[x] on SEA may function as a ligand for DC-SIGN, we investigated the binding properties of these antigens to K562 cell transfectants expressing mutated forms of DC-SIGN. Mutation of either E[324] to Ala, or E[347] to Gln in DC-SIGN (Figure 19a) resulted in complete loss of

5   interaction with both SEA and Le[x], indicating that binding to these antigens is mediated through the primary ligand-binding site and is Ca^{2+} dependent (Figure 19b). This is similar to results reported for binding of DC-SIGN to HIV-1 gp120 [37].

It has recently been demonstrated that a specific mutation in the

10   CRD of DC-SIGN (V[351]G) allows binding of HIV-1 gp120, but abrogates binding to ICAM-3, a T-cell ligand that has been shown previously to interact with DC-SIGN. Our results indicate that the DC-SIGN V[351]G mutant does not bind to either SEA or Le[x], whereas binding to HIV-1 was still observed, indicating that Val[351] is essential for binding to both SEA and Le[x], but not to

15   HIV-1 (Figure 19b). To determine whether the SEA binding site overlaps with the ICAM-3 or HIV-1 binding sites on DC-SIGN, we explored whether SEA inhibits the interaction of DC-SIGN to ICAM-3 and HIV-1 gp120 in a DC-binding assay. The results demonstrate that SEA can inhibit the interaction between DC and ICAM-3 as effectively as the anti-DC-SIGN MAb, whereas

20   binding of DC-SIGN to HIVgp120 was only partially inhibited by SEA (Figure 19c). These results demonstrate that the SEA binding site on DC-SIGN may resemble the ICAM-3 binding site and may partly overlap the binding site for HIV-1 gp120.

25   Example 15. L-SIGN does not interact with DC-SIGN binding SEA or Lewis X

L-SIGN, an adhesion receptor that resembles DC-SIGN by recognizing ICAM-2, ICAM-3 and HIV-gp120, contains a CRD that is nearly identical to that of DC-SIGN[38], and both receptors recognize high-mannose-

30   type N-glycans. Our results above demonstrated a novel binding activity of

DC-SIGN to fucosylated glycans and showed that Val[351] in DC-SIGN is
essential for this binding activity. At the corresponding position in the CRD of
L-SIGN a Ser is present instead of Val (Figure 19a), raising the question
whether L-SIGN can recognize SEA and Le[x]. Binding of L-SIGN to these
5   antigens was investigated by an adhesion assay where fluorescent beads
containing SEA and Le[x]-PAA, respectively, were incubated with transfected
K562 cells expressing recombinant DC-SIGN and L-SIGN (Figure 19e). K562
cells expressing L-SIGN (K-L-SIGN) did not interact with SEA nor with Le[x]-
PAA, but showed interaction with HIV-1 gp120. K562 cells expressing DC-
10   SIGN (K-DC-SIGN) bound to SEA and Le[x], as expected (Figure 19b). These
data show that the two highly related receptors DC-SIGN and L-SIGN have
distinct carbohydrate binding specificity and pathogen-recognition features.

Example 16. Novel carbohydrate specificity predicts and confirms novel
15   cellular counter-structures for DC-SIGN; DC-SIGN recognizes Le[x]
carbohydrates on CD66a present on granulocytes to mediate DC-granulocyte
interactions

DC-SIGN is known to interact with ICAM-2 and ICAM-3, however
20   the glycan ligands on these molecules have not yet been identified. The blood
group antigen Le[x] (CD15) is expressed by gastric mucosal epithelial cells, and
by polymorphonuclear leukocytes (granulocytes) (Figure 20a); indeed DC-
SIGN-Fc-coated beads bind strongly to granulocytes (Figure 20b). Moreover,
DC-SIGN-Fc also binds strongly to granulocytes (Figure 21). The binding is
25   specific for DC-SIGN since it can be inhibited by anti-DC-SIGN antibodies,
mannan and depletion of calcium by EGTA but also by an anti-Le[x] antiobidy
indicating that the cellular interaction is Le[x] dependent (Figure 20 and 21).
Moreover, an antibody against Le[x] specifically reduces the observed adhesion
of DC-SIGN to CD66a, CD66e and CD11b demonstrating that Le[x]
30   carbohydrates indeed participate in the interaction (Figures 20, 54, 59D).

74

Other glycan structures such as Le^y and Le^b can also be involved since the observed block is not complete. This demonstrates that granulocytes express a cell-surface glycoprotein that expresses a glycan structure that is recognized by DC-SIGN.

5   Moreover, we predict that based on the carbohydrate recognition pattern of DC-SIGN (Figure 1), DC-SIGN may also mediate binding of DC to tumor cells, since Le^y expression is increased on many carcinomas including ovary, pancreas, prostate, breast, colon and non-small cell lung cancers[24], while sulfo-Le^a is present on certain tumors that express mucins. Thus, these

10   results indicate that recognition of distinct carbohydrate structures by DC-SIGN may allow DC mediated cell adhesion to T cells, endothelial cells, PMNs as well as to tumor cells.

          Immuno-precipitation studies revealed that DC-SIGN-Fc does not bind the cellular DC-SIGN counter-structures ICAM-2 or ICAM-3 from

15   granulocytes but binds to a novel glycoprotein with a weight of 66kD protein that is only present on granulocytes (Figure 22a). The fact that DC-SIGN binds Le^x carbohydrates and the fact that granulocytes express high levels of CD66a, a Le^x bearing cell-surface receptor with a molecular weight of 66kD (Figure 22b and c), prompted us to examine binding of CD66a-Fc binding to

20   DC-SIGN expressing transfectants and immature DC that express DC-SIGN. Strong binding of CD66a to DC-SIGN expressed by both DC and DC-SIGN transfectants was observed (Figure 23c), similar as was observed for ICAM-3 binding (Figure 23b). Un-coated beads did not interact with these cells (Figure 23a). The interaction of CD66a with DC-SIGN seemed stronger than the

25   previously described ICAM-3-DC-SIGN interaction. These data indicate that CD66a is a novel cellular counter-structure of DC-SIGN and may play an important role in mediating DC-granulocyte cellular interactions that play a role in innate immune responses.

30   Example 17. DC-SIGN binds strongly to a subset of cells in peripheral blood.

To determine whether DC-SIGN preferentially interacts with naïve
T cells expressing high levels of ICAM-3, as was previously postulated, we
started to investigate DC-SIGN binding to total PBL population of cells.
5   Strikingly, DC-SIGN-Fc interacted with a CD3- cell population that were
CD56+ and belonged to NK cell subset (Figure 24). Moreover, the interaction
was concentration dependent (Figure 25) and could be blocked with antibodies
against DC-SIGN (Figure 24 and 25). The CD56+ NK cells in blood can be
divided into two cell populations: CD56^{dim}CD16+ and CD56^{high}CD16- NK cells
10   (Figure 26). DC-SIGN-Fc specifically bound to the CD56^{dim}CD16+ NK
population and the interaction could be inhibited by anti-DC-SIGN antibodies
(Figure 26). ICAM-1-Fc did not interact with the CD56^{dim}CD16+ NK
population, demonstrating that the interaction of DC-SIGN-Fc is specific and
not mediated by the Fc tag. Both NK cell populations were able to specifically
15   interact with ICAM-1-Fc after activation of their β2 integrin LFA-1 (Figure
26). Thus, these results indicate that DC interact with the CD56^{dim}CD16+ NK
cells through DC-SIGN. Strikingly, both NK populations have similar levels of
ICAM-2 and ICAM-3, indicating that DC-SIGN may bind another novel
ligands on the CD56^{dim}CD16+ NK population. Indeed, immuno-precipitation
20   reveals that DC-SIGN-Fc binds, besides ICAM-2, also a molecule with
molecular weight of 166 kDa (Figure 27). This ligand could be CD166 or CD16,
which is heavily glycosylated and only expressed on NK cells.

Example 18. DC-SIGN mediates DC-NK cell interaction and is involved in the
25   lysis of immature DC by NK cells.

To investigate whether the interaction of DC-SIGN with NK cells is
relevant in NK mediated immune response we invested both the lysis of DC by
NK cells and the maturation of DC by NK cells; two processes that have been
30   recently identified to play an important role in innate immunity.

76

DC-SIGN did not inhibit DC maturation by NK cells (Figure 28) but
was strongly involved in the NK-mediated immature DC lysis (Figure 29),
since antibodies against DC-SIGN inhibited the NK-mediated lysis of DC.
These data indicate that DC-SIGN regulates the interaction of immature DC
5   with NK cell by binding a 166 kDa protein on CD56^{dim}CD16$^+$ NK cells. This
DC-SIGN-NK cell interaction also strongly enhances NK-mediated lysis of cell-
lines transfected with DC-SIGN (Figure 30), whereas antibodies against DC-
SIGN inhibit the lysis. These results indicate that DC-SIGN may be important
in the interaction of DC with NK cells and that inhibition of this interaction
10   prevents NK-mediated lysis.

Example 19. DC-SIGN interacts with Herpes simplex virus type 1 and 2

Previous results demonstrated that DC-SIGN specifically interacts
15   with HIV-1[39] and recently it was published that DC-SIGN binds to Ebola
virus[40]. Therefore, we investigated whether DC-SIGN and its homologue L-
SIGN can interact with other viruses, that contain glycosylated envelope
proteins. Strikingly, DC-SIGN-Fc binds strongly to Herpes simplex virus
(HSV)-1 and –2, and this interaction is specically inhibited by antibodies
20   against DC-SIGN (Figure 31). Further analysis demonstrates that DC-SIGN
interact with the HSV glycoprotein gB (Figure 32) since DC-SIGN expressed
by DC and transfectants binds strongly to gB-coated beads. This interaction is
blocked with anti-DC-SIGN antibodies (Figure 32). DC-SIGN is specifically
expressed by DC and L-SIGN is expressed by Liver sinusoidal endothelial cells
25   (LSEC) and some macrophage populations. These data indicate that both DC-
SIGN and L-SIGN could be involved in the interaction of HSV with DC and
LSEC, respectively. Indeed, DC strongly bind to HSV-1 gB and the
interactions are mediated by DC-SIGN, since antibodies against DC-SIGN
block this interaction (Figure 32). Thus, the interaction of DC with HSV is
30   mediated by DC-SIGN and this C-type lectin could be important in the

infection of DC by these viruses. Moreover, these data could suggest that these viruses target DC-SIGN on DC not only to infect these DC but also to evade the immune response by modulating DC function as we demonstrated for mycobacteria and HIV-1.

Example 20

Materials and methods particular to this example and relevant for the experiments described in figure legends 33 to 66.

Bacterial strains.

H. pylori clinical isolates of different geographic origins (The Netherlands, Canada, Poland, Italy, and the People's Republic of China) have been described before[5], as well as *H. pylori* strain NCTC 11637 and phase variants 1c, K4.1, K5.1, 3a, and 1b[9]. *H. pylori* strain 4187E and generation of *futA* (gene HP0379) and *futB* (gene HP0651) knockout mutants have been described[10]. Clinical isolate *H. pylori* J223 was a obtained from Dr. H.P. Wirth [6]. Le$^{x/y}$ positive and negative variants of J223 were isolated as previously described[9]. Briefly, bacteria from the biopsy were inoculated on Dent plates (Columbia agar, 5% horse blood + Dent supplement), followed by one single passage in fluid phase (Brucella broth + 3% fetal calf serum) and distribution over solid media, after which detection of LPS phase variants took place by colony blotting[9]. Genotyping by Amplified Fragment Length Polymorphism (AFLP) analysis was performed as described in reference[39] with the following modifications: : In a final volume of 20 mL, 20 ng of purified chromosomal DNA was digested with 1 U of *Eco*RI (Pharmacia, Uppsala, Sweden) and 1 U of *Mse*I (New England Biolabs, Beverly, MA). The final volume then was increased to 30 mL by addition of 50 pmol each of the *Eco*RI adaptor and *Mse*I adaptor, 1.2 U of T4 DNA ligase (Pharmacia), 1 mM ATP, and ligase buffer.

The adaptors were allowed to ligate to the restriction fragments for 16h at
167C, after which the sample was diluted with distilled water in a final
volume of 500 mL. Texas Red fluorescent-labeled *Eco*O primer (50 -
AGACTGCGTACCAATTC-30 ; Isogen Bioscience, Maarssen, The Netherlands)

5   and unlabeled *Mse*O primer (50 -GACGATGAGTCCTGAG-30) were used for
DNA amplification in a GeneAmp PCR system 9700 thermal cycler (Perkin-
Elmer) with 35 cycles: denaturation (30 s at 94° C), annealing (30 s at 65–56°
C), and DNA molecule extension (60 s at 72° C). In the first 12 cycles, the
annealing temperature was lowered 0.7° C

10   per cycle. After completion of the cycle program, each sample was analyzed
according to the manufacturer's instructions in a Vistra 725 automated DNA
sequencer (Amersham Life Science). Fluorescent AFLP images were stored as
TIFF files with the Vistra 2 TIFF software (Amersham).

15   Monoclonal antibodies and glycoconjugates.

H. pylori strains and phase variants were serotyped as previously described[5]
with the following monoclonal antibodies (mAbs): Hp151, specific for Le[y], 6H3,
specific for monomeric Le[x] and 4D2, specific for H type 1 (all three a kind gift
from R. Negrini, General Hospital, Brescia, Italy). MAb 54.1F6A, specific for

20   polymeric Lewis x (a kind gift from G. van Dam, Leiden, The Netherlands),
NAM61-1A2,specific for i antigen (a kind gift from D. Blanchard, Regional
Blood Transfusion Service, Nantes, France)[10]. MAbs AZN-D1 and AZN-D2[21]
were used to block DC-SIGN and MAb Clone 17 (a kind gift from S. Gordon,
University of Oxford, UK) was used to block the mannose receptor.

25   Synthetic glycoconjugates (Syntesome, Munich, Germany) comprised mono-
and oligosaccharides that were multivalently linked to a polyacrylamide
carrier. Ceramide-linked dimeric-, trimeric and tetrameric Le[x] antigens were a
kind gift from R.R. Schmidt, University of Konstanz, Germany, and were
synthesized as described[40].

79

Cells

Immature DC were generated by culturing monocytes in RPMI-1640/10% FCS
in the presence of IL-4 (500 U/ml, Schering-Plough, Kenilworth, NJ) and GM-
5   CSF (800 U/ml, Schering-Plough) for 5-8 days[41]. K-562 cells, K-562-DC-SIGN
cells and RAW 264.7 macrophages were cultured as described[41].

Bacterial binding

H. pylori cells were labeled with FITC. Cells (50,000) in TSM with 0.5% BSA
10   were preincubated with 20 ug/ml mAb, 200 ug/ml mannan or 10 mM EDTA for
10 min at RT. FITC-labeled bacteria (10 bacteria/cell) were added and
incubated for 45 min at 37°C. Samples were analyzed by flow cytometry.

Soluble DC-SIGN-Fc adhesion assay.

15   The soluble DC-SIGN adhesion assay was performed by ELISA[42]. Antigens
(either 3,75 x 10^{6} bacterial cells/well or 5 μg/ml, in case of LPS or
glycoconjugates) were coated in ELISA plates and soluble DC-SIGN-Fc (1
☐g/ml in TSM) binding was determined by an anti-humanIg-Fc ELISA. When
indicated, DC-SIGN-Fc was preincubated with with 20 ug/ml mAb, 200 ug/ml
20   mannan or 5 mM EDTA for 10 min at room temperature.

Immunohistochemistry

After informed consent by the patients, 2 antral biopsies were collected during
gastroscopy. Tissue cryosections were fixed in acetone and incubated with
25   AZN-D1 followed by anti-mouse-HRP. Staining was performed with the ABC-
AP Vectastain kit and AEC (Vector Laboratories, Burlingame, CA) and
sections were counterstained with heamatoxilin. In parallel, the other biopsy

80

was analyzed for *H. pylori* infection by incubation in urease-medium for 12 hrs. No differences in morphology and staining were observed between non infected and infected individuals.

5   DC activation and Th1/Th2 differentiation

To analyze maturation, DC (180,000 cells) were incubated with H. pylori for 1 hr at multiplicity of infection (M.O.I) of 1, 5, 10, 20, washed and cultured for 20 hrs. Cells were analyzed for maturation markers (CD80, CD86, CD83, HLA-DR) by flowcytometry and supernatant was collected for cytokine ELISA[22].

10   For T cell differentiation, DCs were cocultured with *H. pylori* (M.O.I of 10) for 2 days, washed and incubated with CD45RA+, CD4+ T cells (5,000 T cells/20,000 DCs). In parallel, DCs were analyzed for maturation markers, described above, and cytokine production after stimulation with J558 transfected with CD40L (kind gift from P. Lane, University of Birmingham,

15   UK) in the presence or absence of IFN-□ (1000 U/ml).

Quiescent T cells were restimulated with PMA (10 ng/ml, Sigma-Aldrich, St. Louis, MO) and ionomycin (1 µg/ml, Sigma-Aldrich) for 6 hr, the last 5 hrs in the presence of brefeldin A (10 µg/ml, Sigma-Aldrich). Single-cell production of IL-4 and IFN-γ was determined by intracellular flowcytometry[43].

20

Results

DC-SIGN has been demonstrated to have a carbohydrate specificity for mannose-containing carbohydrates, Lewis antigens and GlcNAc-containing

25   structures (Figure 33, 34, 63-66). We have demonstrated that the carbohydrate specificity of L-SIGN is different since it does not recognize Lewis antigen carbohydrate structures whereas it does recognize high mannose structures (Figure 33 and 34). The murine homologue mSIGNR1 recognizes similar to DC-SIGN high mannose and Lewis antigens, but has also specificity for

sialylated Lewis antigens in contrast to DC-SIGN and L-SIGN (Figure 33 and
34).

DC-SIGN is a C-type lectin in particular highly expressed on Dendritic cells.
Its function is to recognize antigen and process and present this very

5   efficiently into MHC calss I and II molecules.

It has long been thought that DC-SIGN is a pathogen recognition receptor that
recognizes pathogens to activate the immune system, but as it seems now, the
long list of pathogens that target DC-SIGN all persist and escape immunity by
different mechanisms. We have now four examples of pathogens that target

10   DC-SIGN to survive in the host.

1. HIV-1 and HCV target DC-SIGN to ' hide' within DC and escape the
intracellular routing to the lysosomal compartment.

2. Secretion of ManLam by mycobacteria targets DC-SIGN to induce
downmodulation of TLR induced DC maturation, and induction of IL-10.

15   3. the human gastric pathogen *Helicobacter pylori* persists: Lipopolysaccharide
phase variants modulate the Th1/Th2 balance through interaction with the
dendritic cell lectin DC-SIGN.

4. Lactobacillus target DC-SIGN on DC and induce regulatory T cells,
inhibition of recognition of Lactobacillus by DC-SIGN reduces the regulatory

20   state of T cells. It is therefore supposed that DC-SIGN is a receptor that
'normally' recognize self-antigen to tolerize (Annual Review Immunology,
2004). We here demonstrate that targeting of DC-SIGN by lactobacilli can
indeed induce regulatory T cells. Furthermore it seems that DC-SIGN can also
recognize the tumor antigen CEA (CD66e) in particular Lewis X and Y. These

25   tumor antigens may be secreted and suppress DC activation in a similar way
as ManLam of mycobacteria tuberculosis.

The interaction of DC with granulocytes is elucidated by the fact that DC-
SIGN recognizes CD66a and CD11b on granulocytes. In particular Lewis X
antigen on these molecules is recognized. In that way DC-SIGN functions as a

30   cell adhesion receptor that mediates cellular interactions of granulocytes with

DC. This cellular adhesive function between granulocytes and dendritic cells has never been reported but it is likely that the granulocyte DC interaction is essential to form a bridge between the innate immune response and the adaptive immune response in such a way that the granulocyte passes the

5   infectious agents to the DC mounting an adequate immune response. In particular the granulocyte activates and matures the DC, inducing upregulation of cytokines and costimulatory molecules which is necessary for DC migration and initiation of the adaptive immune response. The interaction of DC with granulocytes is also seen in vivo in the situation of Crohns disease.

10   A new pathogen that interacts with DC-SIGN on DC is *Neisseria Meningitidis*. Strain variants demonstrate that in particular one strain mutant IgtB interacts which contains endstending GlcNAc residue. Other experiments (Figure 66) demonstrate the DC-SIGN also recognizes GlcNac.

83

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